

ANTIOXIDANT ACTIVITY OF MAILLARD REACTION PRODUCTS

BY

KAMLASHKUMAR DEVCHAND

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DEDICATION

**This thesis is dedicated to my father, my wife
and my two sons.**

PREFACE

I declare that the experimental work described in this thesis has not been submitted to any other institution and where use has been made of work of others it has been duly acknowledged in the text.


K. DEVCHAND

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ABSTRACT

The use of natural antioxidants to improve the oxidative stability of food lipids has received special attention because of the worldwide trend to avoid the use of synthetic food additives. A wide range of natural sources has been shown to contain antioxidant properties, these include plant extracts, herbs and spices, citrus fruits, oilseeds and legumes. Some antioxidants have been found to be formed during the heat processing of foods, including the Maillard reaction products that are formed by the reaction of amino acids, peptides and proteins with reducing carbohydrates.

A study was undertaken to investigate the antioxidant activity of Maillard reaction products formed during extrusion of soyabeans. A preliminary oxidation study carried out to identify a suitable substrate revealed that sunflower oil stripped of antioxidants was a suitable substrate with a low induction period of 15 minutes via the Rancimat Method and 4.5 hours via the method of Ross and de Muelenaere. Methyl linoleate was found to be sensitive to oxidation, but not readily available and costly.

Storage test of antioxidant stripped sunflower oil under various headspace conditions showed that the substrate stability was best at 4°C under nitrogen or vacuum. Under such conditions the product could be stored for a period of 136 days. Nitrogen was chosen as the most suitable for this exercise as it was not easy to remove all residual air from the samples by vacuum. Furthermore with nitrogen headspace residual O₂ could be measured based on N₂/O₂ ratio changes. Hexane solvent was found to be able to remove all lipids from soyabeans.

Under the experimental conditions practised it was found that the induction periods for extruded and unextruded soya flour hexane extracted lipids were very similar. Addition of glucose or fructose to the extrusion mixture increased induction period of hexane extracted lipids by 37.5% and 1.5% respectively as measured by the Ross and de Muelenaere method and by 50% and 6.5% respectively as measured by the Rancimat Method. Available lysine of glucose containing extrudate was reduced

by 69% while that of the fructose containing extrudate was reduced by 23%. Residual glucose and fructose analysis of extrudates showed that 66% of glucose was utilized in the formation of the Maillard reaction products while only 21% of fructose was utilized during extrusion processing.

Comparison of induction periods of soya glucose and soya fructose extrudates to induction period of TBHQ antioxidants (200ppm) in antioxidant stripped sunflower oil gave antioxidant activity of 86ppm and 9ppm for soya glucose extrudates and soya fructose extrudates respectively.

The observed antioxidant activity of Maillard reaction products could be utilized with success in different types of processed foods without the need for extensive testing as required for synthetic antioxidants but supplementation of lysine may be required to maintain nutritional balance.

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LIST OF ABBREVIATIONS

MRP	Maillard Reaction Products
IP	Induction Period
AH	Antioxidant
BHA	2-or 6-tert-butylhydroxyanisol
BHT	2-6-di-tert-butylhydroxytoluene
ARP	Amadori rearrangement product
TVP	Textured Vegetable Protein
TBA	Thiobarbituric Acid
AOM	Active Oxygen Method
OSV	Oxidative Susceptibility Value
SAD	Series Across-Detector
GSC	Gas Solid Chromatography
ID	Internal Diameter
TCD	Thermal Conductivity Detector
AR	Analytical Reagent
FID	Flame Ionisation Detector
Degs - PS	Diethyleneglycol Succinate-Phosphoric acid
IV	Iodine Value
UFA	Unsaturated Fatty Acid (s)
N ₂ /O ₂	Nitrogen to Oxygen
FFA	Free Fatty Acids
PV	Peroxide Value
FDNB	2-4 Dinitrofluorobenzene
DNP - lysine	Dinitrophenol - lysine
HPLC	High Pressure liquid Chromatography
DF	Double Flight
SF	Single Flight
R	Reverse
T.B.H.Q.	tert-butylhydroquinone
Aw	Water activity
MRV	Maillard reaction volatiles

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CHAPTER 1

INTRODUCTION

There is a general trend towards substituting synthetic antioxidants in foods by the addition of natural antioxidants and natural oxidation inhibitors or by the preferential use of natural ingredients that develop antioxidant activity during food processing. Such antioxidants have been found to be formed during heat processing of foodstuffs by the reaction of amino acids, peptides and proteins with reducing carbohydrates to form Maillard reaction products.

Maillard reaction products (MRP) contribute highly desirable flavours to heated foods such as bread, toast, cereal products and meats. Low molecular weight carbonyl compounds produced by the Maillard reaction condense with amino acids and other amine derivatives to form heterocyclic products such as dihydropyridines that have been shown to have strong antioxidant activity in model systems and which can deactivate singlet oxygen. Many Maillard reaction products such as premelanoidins and melanoidins which have antioxidant properties have been successfully utilized in different types of foods. Their activity involves the reducing capacity of the carbonyl compounds present in the melanoidins. Such products also have the ability to inhibit nitrosamine formation, reduce nitrosamine induced carcinogenicity and scavenge active oxygen (1).

Extrusion-cooking that takes place at comparatively low moisture content is known to favour the Maillard reaction. Materials containing low levels of reducing sugars are less prone to deterioration during heat treatment. However, under extreme extrusion conditions, formation of reducing carbohydrates occur through hydrolysis of sucrose or starch to form glucose which reacts with free amino groups of lysine or other amino acids to form Maillard reaction products (2). Under very mild extrusion conditions no reducing sugars occur therefore no Maillard reaction products are formed and no increased antioxidant activity is observed, except upon addition of reducing sugars. Such sugars are normally added to cereals, biscuits and baked products to promote the browning (Maillard) reaction (2).

The present work involves a study on how such antioxidant reactants are formed during mild extrusion conditions and how components of the extrusion mix can influence the induction period on an antioxidant free oil substrate. Methods to measure such effects will be studied, as well as the determination of a suitable substrate to be used in the study.

CHAPTER 2

LITERATURE REVIEW

2.1 LIPID AUTOXIDATION

Lipid oxidation spontaneously occurring in foods is referred to as autoxidation which is a complex process and affects many aspects of food quality. Flavour deterioration is one important effect, but impaired colour and texture are also common consequences of lipid autoxidation, as are losses in nutritional value such as reactions with vitamins. The main targets of oxygen attack are unsaturated fatty acids but also other compounds such as sterols, carotenoids, and aroma compounds can also be involved. The reaction between oxygen and lipids is initiated either by the formation of free radicals from the lipids or by the formation of active oxygen species that react directly with the lipids (3). The rate of autoxidation is affected by fatty acid composition, degree of unsaturation, the presence and activity of pro- and antioxidants, partial pressure of oxygen, the nature of the surface being exposed to oxygen and the storage conditions (temperature, light, moisture content, etc.) of fat/oil-containing foods (4). One of the characteristics of autoxidation is the Induction Period (I.P.) of a fat, for example the oxidation of lard (Figure. 2.1). Induction Period is the time required prior to oxidation during which oxidative products are being produced at an accelerated pace (5).

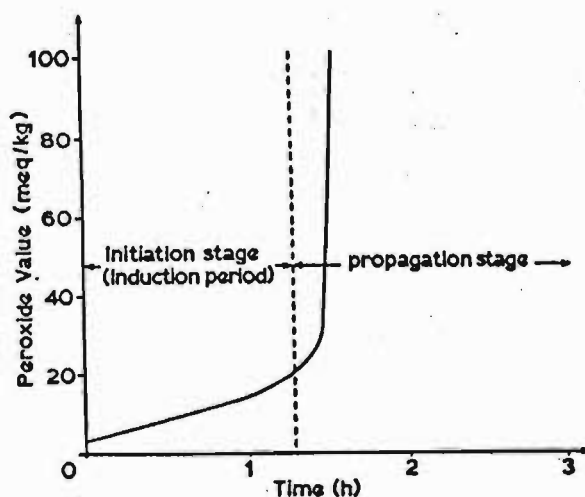
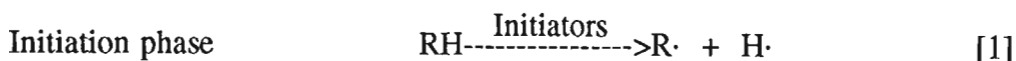


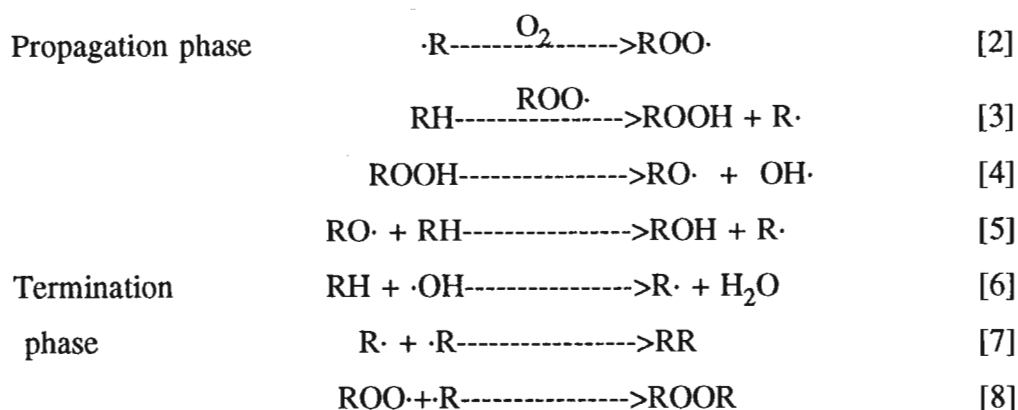
Figure 2.1 Oxidation of lard (5).

Unsaturated fats and oils undergo autoxidation when the unsaturated portions of the fatty acid residues of the triacylglycerols react with molecular oxygen to form peroxides, hydroperoxides, and carbonyl compounds. The hydroperoxides split into smaller short chain organic compounds such as aldehydes, ketones, alcohols, and acids which are responsible for the off-odours and flavours characteristic of rancid fats and oils (6).

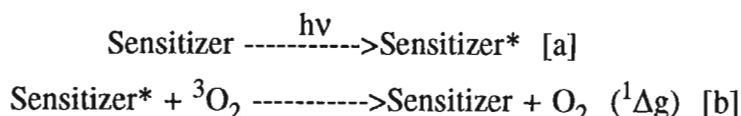
Several conditions that will catalyze autoxidation are heat, light, heavy metals, such as iron and copper, alkaline conditions, alkaline metals, chemical unsaturation and the presence of residual pigments and oxygen (6). Autoxidation is accepted to be a free radical process, and in common with all free radical reactions, the length of the induction period is sensitive to the presence of minor components which either inhibit the formation of free radicals and extend the induction period (antioxidants) or shorten the induction period (prooxidants) (5).

The autoxidation of unsaturated fatty acids can be divided into three phases, namely, initiation, propagation and termination, as indicated in equations [1] to [8]. Initiators, such as energy (light, heat), traces of heavy metals, and peroxides attack the substrate RH and produce highly reactive free radicals ($R\cdot$) [1]. During propagation free radicals react with oxygen to produce peroxide radicals ($ROO\cdot$) [2]. The peroxide radicals have the ability to attack another fatty acid RH, which results in a hydroperoxide ($ROOH$) and a free radical ($R\cdot$) [3]. Peroxides are unstable compounds which are again decomposed to radicals, aldehydes, ketones, and alcohols, that is reactions [4] and [5]. The volatile decomposition products are responsible for the off-odours. During termination, the quantity of highly reactive compounds rises constantly until they begin to interact, that is reactions [6], [7] and [8], then the concentration of radicals and peroxides falls. Stable deterioration products are formed (7).





The free radical mechanism of autoxidation is well established with regard to the initial reaction [1] where the initiating species that give rise to the first free radical ($R\cdot$) involve short-lived singlet oxygen 1O_2 species. Hydroperoxide formation as a result of reaction of oxygen with lipids requires a change in total electron spin as the hydroperoxide and lipids are in the singlet state while oxygen is in the triplet state 3O_2 (8). Conversion of triplet oxygen to singlet oxygen is effected by sensitizers such as chlorophyll, pheophytin, myoglobin and riboflavin in the presence of copper and light (9). The sensitized photo oxidation steps can be summarised as follows.



The electronically excited sensitizer, produced upon light absorption [a] transfers energy to ground-state oxygen and $O_2 ({}^1\Delta_g)$ is produced [b] (10).

Lipid autoxidation can only be completely prevented by the total exclusion of oxygen, but it can be slowed down by the addition of inhibitors which would increase the induction period. Inhibitors of autoxidation may be classified into groups according to their mechanism of action as listed below (11).

<u>Type of inhibitor</u>	<u>Mechanism of action</u>
Antioxidants	Reaction with free radicals, interrupting the propagation phase of the chain reaction
Synergists	Increasing antioxidant activity of primary antioxidants
Retarders	Reducing hydroperoxides without forming free radicals
Metal scavengers	Inhibiting the ability of heavy metals to catalyse the production of free radicals
Singlet-oxygen quenchers	Deactivating singlet oxygen, which may initiate the free radical chain reaction

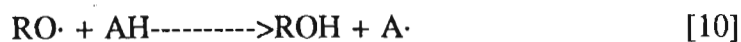
Antioxidants inhibit or interfere with the autoxidation process by competing for the free radical formed in reaction [3].



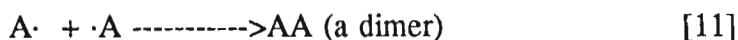
Antioxidant (AH) reacts with the peroxy radical to form a lipid hydroperoxide and a stable radical A \cdot .



The alkoxy radicals also react in a similar way



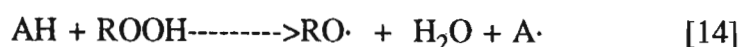
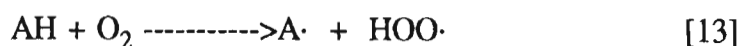
The antioxidant free radical, A \cdot , is not active to propagate the free radical reaction chain and is usually deactivated by combining with another A \cdot radical to form a dimer (stable inactive product)



In some cases the antioxidant may react directly with the free radical R \cdot , usually in the presence of quinones



The ability of antioxidants to do this is based mainly on their phenolic structure. Antioxidants (AH) function by donating a hydrogen atom to the fat free radical (R \cdot) to reform the fat molecule (RH) and a stable antioxidant free radical (A \cdot). Under very high antioxidant concentrations the antioxidant may behave as a prooxidant [13] and [14].



Autoxidation can be retarded or inhibited by adding a low concentration of a chain-breaking antioxidant (AH) which can interfere with either chain propagation or initiator free radicals. The inhibition depends on the activity and structure of the antioxidant, on the rate of chain initiation and on antioxidant concentration. The critical concentration of an antioxidant is its concentration in the system required to interrupt all oxidation chains. During the initial oxidation process the antioxidant is gradually consumed by reacting with free radicals present. When practically all the antioxidant has reacted, oxidation proceeds in the usually autocatalytic way. Thus, the addition of an antioxidant results in an extended induction period during which very little oxidation takes place (12).

2.2 NATURAL ANTIOXIDANTS.

In the early 1980s, food manufacturers began looking for "natural" ingredients in their foods in view of consumer opposition to synthetic antioxidants. This trend has promoted extensive research in the area of natural antioxidants. As a result increasing interest has been shown in such materials as tocopherols, rosemary extracts, spices, herbs, tea, oil seeds, cereals, grains, fruits, vegetables, proteins, and protein hydrolysates. The reactive chemical moiety in most natural antioxidants is the same as that in synthetic antioxidants, for example BHA and BHT, an aromatic ring with at least one hydroxyl group (13). The phenolic antioxidants act as free radical scavengers. There are also antioxidants that are oxygen scavengers or reducing agents or act as chelators binding metal ions (13). In addition other types of naturally occurring chemicals with antioxidant activity have been identified. Some of these types of antioxidants have been found to be formed during the heat processing of foods. These include Maillard reaction products formed by reaction of amino acids, peptides and proteins with carbohydrates (14). From the list given below it is evident that many food ingredients possess natural oxidation inhibitors but some such foods are of limited use as they often impart non specific flavour, aroma, and colour to the finished product. Furthermore those

that have low antioxidant activity or low solubility in fats and oils are of limited use (11).

List of sources of natural oxidation inhibitors.

- a) Oils and oilseeds
Tocopherols and tocotrienols; sesamol and related substances; olive oil resins; phospholipids
- b) Oat and rice brans
Various lignin-derived compounds
- c) Fruits and vegetables
Ascorbic acid; hydroxycarboxylic acids; flavonoids; carotenoids
- d) Spices, herbs, tea, cocoa
Phenolic compounds
- e) Proteins and protein hydrolysates
Amino acids; dihydropyridines; Maillard reaction products.

2.2.1. SOURCES OF NATURAL ANTIOXIDANTS.

a) **Tocopherols and related compounds.**

Tocopherols occur as minor constituents in vegetable oils and are among the best known and most widely used antioxidants. The α -, β -, γ -, and δ - tocopherols differ in the degree of methylation of the dihydrochromanol ring. Their antioxidant activities depend very much on the food to which they are added, the concentration used, the availability of oxygen and the presence of heavy metals and various synergists (12). At high concentration and in the presence of trace levels of iron and copper salts, tocopherols may in fact act as prooxidants. An increase in concentration of α -tocopherol from 0.4% to 4.0% and in the presence of traces of iron causes a conversion of its antioxidant activity to a prooxidant activity. The prooxidant activity is caused by the formation of radicals during oxidation by gaseous oxygen (15). Satisfactory antioxidant activity is obtained only when tocopherols are used in combination with synergists such as ascorbic acid, citric acids, and some

amino acids such as cysteine, tryptophan, and lysine or with chelating agents such as citric acid and sodium pentapolyphosphate (14). Their antioxidant activity gradually decreases from δ - to α -tocopherol (δ -tocopherol most effective > γ -tocopherol > β -tocopherol > α -tocopherol least effective). They work as antioxidants by donating the hydrogen of the hydroxyl group to a fatty peroxide free radical (16).

b) **Antioxidants from sesame oil and olive oil.**

Antioxidants from sesame oil, which by itself is quite stable to oxidation, are produced from lignins in the seeds via precursors such as sesamol, which is hydrolysed on heating to sesamol (11).

Olive oil is very stable, not only because of its low content of polyenoic fatty acids, but also due to the presence of various natural bitter-tasting antioxidants mainly derived from hydroxytyrosol (a derivative of pyrocatechol) which is a product of tyrosine degradation and other polyphenols (11).

c) **Phospholipids.**

Phospholipids are believed to be major lipid components responsible for the development of off-flavours and odours in a number of food products during prolonged storage. However, because phospholipids contain phosphorus, a nitrogen-containing moiety and polyunsaturated fatty acids (PUFA), their role as pro- or antioxidants in oxidation systems is far more complex than that of the neutral lipids. The phosphorus and nitrogen-containing moieties are possibly involved in stabilizing lipid systems, whereas the PUFA moiety is suspected of destabilizing lipids (17).

Antioxidant properties of phospholipids has been demonstrated through their addition to processed vegetable oils and animal fats, including those from sunflower, corn, cotton seed, soyabean and lard (17). Though the exact

mechanism of action of phospholipids is still not fully established, four postulates have been proposed to explain their antioxidant activity:

- i) synergism between phospholipids and tocopherol (18).
- ii) chelation of pro-oxidant metals by phosphate groups (19).
- iii) formation of Maillard-type products between phospholipids and oxidation products
- iv) action as an oxygen barrier between oil/air interfaces (20).

Phospholipids especially phosphatidylethanolamine and phosphatidylserine showed enhanced antioxidant activity in perilla oil with enriched tocopherols while no effect was observed with phosphatidylcholine. All three phospholipids showed no antioxidant activity when no tocopherols were present in the perilla oil. Without the phospholipids, phosphatidylethanolamine and phosphatidylserine, the high concentration of tocopherols showed prooxidants activity. The antioxidant synergism between tocopherols and phospholipids is closely related to the effects of phospholipids on suppressing the oxidative decomposition of tocopherols (18) and tocotrienols (21).

d) **Antioxidants from herbs, spices and algae.**

The antioxidant activity of various spice extracts have long been recognized and from a study of 107 plant spices more than 50% showed some antioxidant activity. Oregano was shown to contain various antioxidant pyrocatechol derivatives and flavonoids (22). Efficient antioxidants were also found in sage (23), thyme, clove (active component eugenol), ginger, catnip and ginseng (11).

The antioxidant activity of rosemary extracts has been known for about 30 years, and active compounds have been elucidated. The main active substance is carnosol, an active diphenolic diterpene, and its quinone derivative have also been isolated from rosemary leaves (24). Rosmanol also a diterpene with a structure similar to carnosol was isolated from rosemary leaves (25).

Other compounds isolated from rosemary extracts with strong antioxidant activity were Rosmariquinone and Rosmaridiphenol (26). The diterpenes eperosmanol and isorosmanol also have antioxidant activity. Extracts from algae such as *Chlorella SPP* and *scenedesmus acutus* contain various antioxidant compounds (11).

e) **Flavonoids and Carotenoids.**

Flavones and related compounds occur widely in many plant foods. Catechin and quercetin, flavonoid pyrocatechol derivatives, possess high antioxidant activity and have been used to stabilize lard (27). The polyhydroxy - dihydrochalcones are efficient antioxidants found in cabbages, peppers, soyabeans, peas, peanuts, cocoa beans, cottonseed and many other plants (11). Flavonoid pyragallol derivatives such as tea tannins also possess antioxidant activity (27). Tea flavonoids are of practical interest as tea dust grounds are available in large quantities for the preparation of extracts (11).

Plant extracts containing carotenes and various carotenoids which are used as natural colouring materials, also possess antioxidant activity. In the presence of light they act by quenching singlet oxygen produced during photosynthesis (28). The presence of carotenoids in edible oils also helps to protect against the formation of singlet oxygen by blocking light transmission through the oil. The 4-oxo carotenoids such as astaxanthin and canthaxanthin are able to scavenge free radicals (11).

f) **Natural antioxidants from cereals.**

Oat flour and oat extracts were among the first antioxidants proposed for use in the stabilization of fats, oils and fat containing foods. Many patents were taken out on the utilisation of oatmeal as an antioxidant. Oat flour was added to lard at levels ranging from 1% to 10% to protect against oxidation (29). Effective stability of refined corn and soya bean oil was observed at 7.5% addition of oat flour (29). Recent studies on oat oil and its polar fractions revealed inhibition of autoxidation when tested on lard, tallow and

soya bean oil (30). The antioxidant activity of oat flour and oat extracts has been attributed to esters of caffeic and ferulic acids. Similar compounds such as oryzanol (an ester of ferulic acid and a triterpenic alcohol) was found in the brans of other cereals (31). Other active substances identified include flavonoid glycosides (from rice hulls) and esters of sinapic and protocatechuic acids (from rapeseed hulls) (11).

Some sterols present in oats have been shown to retard thermal changes at frying temperature. These sterols have a side chain containing an ethylidene group. Free radicals from the heated oils react with the ethylidene group on the sterols to produce an allylic free radical, which can interrupt the oxidation chain (32). β -sitosterol and Δ^5 -avenasterol were the major sterols present in oats and it has been demonstrated that Δ^5 -avenasterol is the effective agent in reducing changes in soya bean oil during heating (33). The addition of sterols may be a natural alternative to the use of antioxidants in frying oils (32).

Most of the above-mentioned antioxidants from cereals originate from lignins, which are present in many plants. Their metal-scavenging abilities contribute to their stabilizing effects (11).

g) **Polysubstituted organic acids.**

Polysubstituted organic acids, such as citric acid, have been used for the stabilization of fats and oils (11). Decomposition products formed during the heating of citric acid, and other hydroxycarboxylic acids such as tartaric, malic and isocitric acids also possess antioxidant activity. Most hydroxycarboxylic acids act as metal scavengers, and stimulate the decomposition of hydroperoxides such that no free radicals are formed (11). Ascorbic acid and its synthetically produced esters are widely used as synergists for the stabilization of fats and oils (34).

h) **Proteins, peptides and amino acids.**

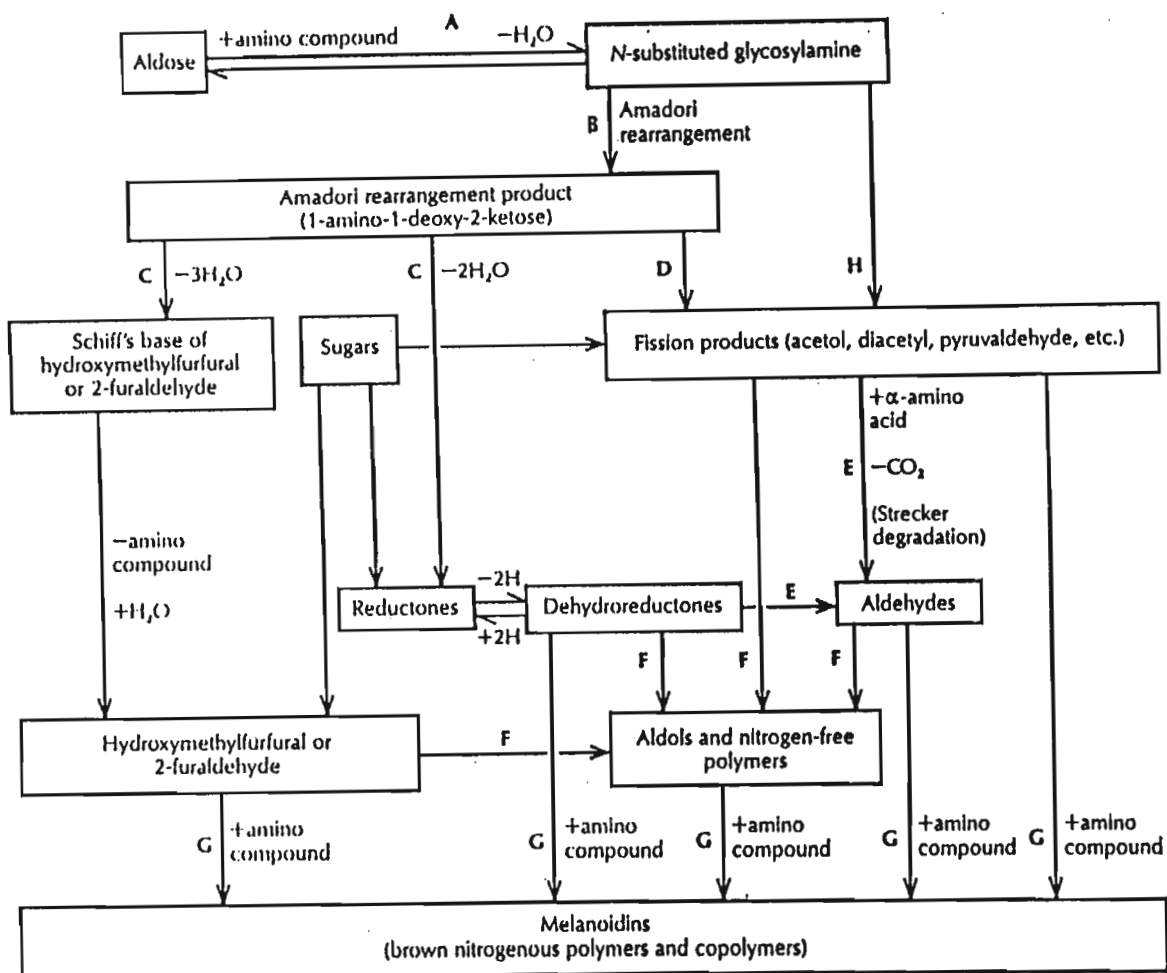
Proteins, peptides and amino acids decrease the rate of autoxidation and the hydroperoxide content of fatty foods. Amino acids are effective synergists in combination with phenolic antioxidants, acting both as heavy metal scavengers and as promoters of hydroperoxide decomposition.(11) Amino acids are particularly suitable for the protection of unsaturated lipids in freeze-dried emulsions where autoxidation is quite rapid. The thermal degradation of phenylalanine gives rise to a number of alkyl aromatic compounds, some of which have been reported to inhibit lipid oxidation.(35) Amino acids and lower peptides present in protein hydrolysates display antioxidant activity in freeze-dried lipid emulsions. Carnosine, a dipeptide of α -alanine and histidine, has been shown to inhibit iron-catalyzed oxidation of muscle foods.(13) A variety of proteins such as wheat gliadin, maize zein, ovalbumin, and soya protein isolate also exhibit considerable antioxidant activity. Gliadin was proven to be the most effective. Its activity is attributed to the high concentration of non-polar amino acid residues such as glutamine, proline and leucine present. The amide group of the amino acid residues play an important role in intra- or intermolecular association via the hydrogen bond.(36) Gliadin antioxidant effectiveness increases at higher water activity, hence the water in gliadin may retard autoxidation not only by hydration of "trace" prooxidants but also by direct action on hydroperoxides or free radicals in cooperation with functional groups of the protein.(36)

2.3 **MAILLARD REACTION PRODUCTS.**

The Maillard reaction is a type of non-enzymic browning of fundamental interest to food chemists and food processors. It largely stems from a desire to produce and control aromas and flavours obtained on baking, cooking, roasting and grilling.(37) The Maillard reaction involves the reaction of carbonyl groups with free amino groups while caramelization occurs in the absence of amino compounds.(38) However, the Maillard reaction while beneficial under certain circumstances is sometimes undesirable, that is when dehydrated foods darken and develop off - flavours on storage or during the heat treatment. The Maillard reaction may also result in a reduction in nutritional value. The development of

antioxidant properties and the formation of potentially toxic compounds is also known to occur.(39)

An outline of the Maillard reaction is given in the following scheme illustrating clearly that it is a complex network of reactions.(39)



The reaction involves the condensation of the carbonyl group of a reducing sugar (aldose) with a free amino group of a protein or an amino acid to give an N-substituted glycosylamine (step A) which rearranges to form the Amadori Rearrangement Product (ARP) (step B). The ARP dehydrates to form furfurals and reductones (step C) and fission (dicarbonyl) products (step D). These compounds, and aldehydes formed by Strecker degradation of amino acids (step E), may react either in the absence of amino compounds to give aldols and high molecular weight nitrogen free polymers (step F) or, in the presence of amino

compounds, to give brown nitrogenous polymers called melanoidins (step G). Step H illustrates a direct route to fission products from N-substituted glycosylamines without the formation of an ARP. Some steps on the formation of Maillard reaction products are well defined (step A-E) while the reactions leading to the development of non - volatile colour compounds (steps F and G) remain obscure.(39) The various Maillard reaction products formed depends on the reaction conditions, the reaction time, the temperature, the concentration of the reactants and the pH.(40)

An increase in temperature increases the rate of development of Maillard reaction products. An increase in reaction time of heating results in increased colour development.(39) Pentose sugars (eg. ribose) react more readily than hexoses (eg. glucose) which in turn are more reactive than disaccharides (eg lactose). In addition individual sugars within each of these groups exhibit different reaction rates, in particular aldoses, such as glucose, behave differently to ketoses such as fructose.(41) Of all the amino acids lysine results in the most colour development in the Maillard reaction due to its ϵ - amino group while cysteine results in the least colour development. Thus food containing proteins that are rich in lysine (eg milk proteins) are likely to brown readily. The sugar to amino compound ratio also influences the amount of colour produced.(42) The Maillard reaction occurs most rapidly at intermediate water activity (A_w) values (0,5 - 0,8). A_w is of most significance to the reaction in dried and intermediate moisture foods.(43) Low pH values (< 7) favour the formation of furfurals from ARP's while the routes to reductones and fission products are preferred at a high pH (> 7). (39)

Extruders are high temperature food reactors which can process on a continuous basis high protein materials into palatable foods. Improvements in functional characteristics of proteins may be achieved through modification of temperature, screw speed, moisture content, and other extrusion parameters. Extrusion can improve the digestibility of proteins, while reducing gossypol,

proteinase inhibitors, allergens, aflatoxins, and other undesirable compounds (44). Extrusion has become the major processing method for textured vegetable protein (TVP), ready-to-eat breakfast cereals, animal feeds, snack foods, and more recently processing of various dairy and meat products (44). Temperature and shear conditions occurring during extrusion provide the chemical and physical means whereby complex starch and proteins fed into the extruder can be partially degraded to provide reactants that can participate in numerous mechanisms to produce Maillard reaction products. Typical reactants include reducing sugars, aldehydes and ketones, amines, amino acids, peptides and proteins (44). These reactants condense to form the brown colours of extruded products and the degree of browning depends on the processing conditions and nature of ingredients (45).

Most studies of the changes that protein undergoes during extrusion processing have concentrated on the free amino groups of lysine, and it has been established that the concentration of these groups decreases following extrusion (46). The amount of available lysine, that possess the free amino group decreases by up to 50% depending on the severity of the processing and the other ingredients present in the extrusion mix (45).

Maillard reaction products have been reported by several authors to inhibit lipid oxidation in model systems as well as in food products. The antioxidative compounds formed have not been identified so far (47). Model reactions between sugars and amino compounds were early shown to yield products with antioxidative effect in foods. A British patent (Borden Co. no. 886519, 1962) is based on a method to produce antioxidative compounds through heating of sugar and protein in a fat medium. "Warmed - over Flavour" formation in cooked meat and turkey could be avoided if Maillard reaction components such as glycine and glucose, lysine and glucose, leucine and glucose, and glycine and lactose were added to meat before heat treatment (48).

In model system studies between amino acids and sugars both histidine and glycine when reacted with xylose gave antioxidative xylose reaction products. In application studies it was found that free histidine and free glucose added to cookie dough retarded the development of rancidity and the formation of hexanal in stored cookies baked from the dough. The effect was greater than that obtained from synthetic antioxidant while on addition of a preformed MRP of histidine and glucose the effect was not as great as that obtained by the individual components. Analysis of retained histidine in the cookies showed that 70% of the added histidine had reacted with glucose to form the anti-oxidative compounds in the cookies during the baking (50).

Melanoidins produced by reaction of reducing sugars with amino acids were good inhibitors of linoleic acid oxidation and acted as synergists with β -, γ - and δ - tocopherols but not with α -tocopherol (49).

The antioxidative effect of Maillard reaction products have been established in many linoleic acid emulsions and storage experiments with various cookies, milk powders and frozen sausages (51). The reaction of Maillard reaction products with oxygen seems to involve the formation of some volatile carbon compounds, possibly carbon dioxide or a similar product. Since the Maillard reaction product reacts with free oxygen it could be that one of the mechanisms for the oxidative effect is simply that the Maillard reaction products are more readily oxidised than the lipids, making oxygen unavailable for the lipids competing for oxygen (47). Several authors concerned with the mechanism of antioxidative action of Maillard reaction products postulate that the products are also effective in reducing peroxides and inactivating radicals as well as in complexing of heavy metals (52). By fractionation of MRPs it became evident that low molecular mass products significantly contribute to the overall antioxidative effect. No correlation between the extent of browning and antioxidative activity has yet been established, the opinion seems to be that in fact colourless intermediates of the

Maillard reaction products essentially contribute to the antioxidative effect of Maillard reaction products (52).

2.4 **METHODS TO EVALUATE ANTIOXIDANT ACTIVITY.**

The efficacy of natural antioxidants and the oxidative stability of edible oils and food emulsions have been difficult to evaluate in view of the questionable conditions and methodology used to follow oxidation. The methodology used to evaluate natural antioxidants must be carefully interpreted depending on the conditions of oxidation and the analytical method employed to determine the extent and end-point of oxidation (53).

The literature on stability evaluations of food lipids is extensive. However, the published data comparing the effectiveness of various antioxidants are often difficult to interpret because of this questionable methodology, particularly the choice of methods utilizing inappropriate oxidation conditions. Natural antioxidants have been especially difficult to evaluate because of the use of crude extracts, and the role of complex interfacial phenomena in oils and food emulsions has further compounded the analytical problems (54).

In order to determine the activity of an antioxidant it is necessary to determine the induction period of a fat with and without the antioxidant. Antioxidants are only effective if present in the fat before the end of the induction period or prior to the commencement of oxidation, and the induction period ends when the antioxidant is consumed (55). The induction period is measured as the time required to reach an end-point of oxidation corresponding to either a level of detectable rancidity or a sudden change in the rate of oxidation. Measurements of induction period under standard conditions are generally used as an index of antioxidant effectiveness. For practical purposes the predictions of oxidative stability in foods and oils based on measurement of induction period should be related to measured product shelf life (53).

Heating is the most common and effective means of accelerating oxidation. In the presence of antioxidants, the activation energy of lipid oxidation is increased because antioxidants lower the rates of oxidation reactions by increasing the overall energy of activation (55). An Arrhenius plot of \log . (overall reaction constant) versus $1/T$ shows that the antioxidant effectiveness increases as T (the temperature) decreases, thus the overall protection predicted at high temperature for an antioxidant will usually be less than that found at lower temperatures (56). Therefore the temperature coefficients are different for a natural fat containing low levels of antioxidants and the same fat containing added antioxidants. The order of activity and ranking of different antioxidants depend on whether they are tested at high or low temperatures. The latter criterion requires testing at several different temperatures (56). Although testing stability under ambient conditions may approximate real storage conditions of foods, the procedure is too slow to be of practical value. Furthermore under slow oxidation the reproducibility of results is compromised by many variables that are difficult to control over prolonged storage periods (53).

Methods using light and metal - catalysed oxidation provide rapid screening tests. However in the presence of sensitizers the mechanism of photo-oxidation is different from that of the free radical autoxidation that usually occurs in foods. Photo-oxidation results in the formation of different flavour precursors with different volatile breakdown products and flavour significance. Oxidation catalysed by metals may result in a higher proportion of breakdown carbonyl products relative to the level of primary hydroperoxides (53).

The weight-gain method, based on an increase in weight due to oxygen absorption is not very sensitive. The end-point requires a level of oxidation that is beyond the point where flavour deterioration is detectable in polyunsaturated oils (57). The Schaal Oven Test for estimating resistance to oxidation involves heating 50g-100g of sample in a open dish and holding this

in a thermostatically controlled oven until rancidity starts. The temperature at which the fat is held is between 60°C to 70°C. The sample is examined at regular intervals and the condition of the fat is determined either by smell and taste or, alternatively, the course of the oxidation can be followed by determination of chemical factors such as the Peroxide Value (54).

Sensory methods based on odour and flavour evaluations provide the most useful information related to consumer acceptance of the food product. Although these methods are sensitive they are highly dependant on the quality of training the taste panel received. The scoring by taste and odour may vary from laboratory to laboratory. Analysis of volatiles by gas chromatography is closely related to flavour evaluations and is therefore the most suitable for comparison with results of sensory panel tests (58).

The thiobarbituric acid (TBA) method is based on the colour reaction between TBA and oxidation products of polyunsaturated lipids. The test is specific and relates to the level of aldehydes present in the oil (54). TBA values may overestimate the extent of oxidation since other components such as Maillard reaction products, protein and sugar degradation products interfere with the formation of the TBA colour complex (53).

The spectrophotometric method for carotene bleaching by co-oxidation of linoleic acid is simple and sensitive but not specific and is subject to interference from oxidizing and reducing agents present in crude extracts. Furthermore linoleic acid is not an appropriate substrate since food lipids are mainly triglyceride in nature (59).

Oxygen absorption methods have limited sensitivity and require high levels of oxidation at the end-point for measurement of induction periods. Determination of Peroxide Value (P.V.) provides an empirical measure of lipid oxidation that is less sensitive and precise than sensory and headspace methods for volatiles. The information provided by both oxygen absorption and P.V.

methods is related to the amounts of hydroperoxides that are readily decomposed at temperatures above 60°C (53).

The Active Oxygen Method (AOM), the Swift test and the Rancimat test are tests in which the oxidative deterioration of a fat is accelerated not only by the use of an elevated temperature (usually 100°C) but also by bubbling air through the sample. However the activity of volatile antioxidants such as BHA (butylated hydroxyanisole) is underestimated by these tests as the antioxidant is lost from the samples by evaporation under these temperatures (55). The AOM has been standardized in the Official and Tentative methods of the American Oil Chemist's Society Method Cd 12-57 and involves exposing the fat sample to a stream of dry air at a temperature of 100-140°C. The progress of the oxidation curve can be followed by periodic determinations of Peroxide Value or other parameters. The curves consist of an induction phase, in which practically no secondary products are formed, and an oxidation phase during which a large increase in Peroxide Value and volatile products is detected (55). The Rancimat method is an automated method which utilizes this fact. The greater part of the volatile products consists of formic acid and the method relies on continuous monitoring of the electrical conductivity of the aqueous effluent (60). Results of the induction periods determined using the Rancimat method have been shown to correlate extremely well with those of the Active Oxygen Method and Oil Stability Index (OSI) (61).

2.5 **SUBSTRATE SELECTION.**

Much work has been published in current and early literature comparing the effectiveness of antioxidants with different lipid substrates, model systems, oxidation conditions, and methods to determine lipid oxidation. The diversity in oxidation conditions and methods used to determine lipid oxidation has led to considerable confusion. Natural antioxidants have been difficult to evaluate in oils, food emulsions and model substrates in view of the complex interfacial affinities between air - oil and oil - water (62).

In research studies, stability tests to evaluate the extracted natural antioxidants often use different isolated lipid substrates. The efficacy of natural antioxidants or their synergistic effect is dependent on substrate and storage conditions. The substrate may consist of an oil along with other interacting ingredients that make up the formulation in which the antioxidant is to be utilized (62).

Current research findings indicate significant differences in the performance of antioxidants when tested with the customary methyl linoleate, linoleic acid emulsions and other standard methyl esters when compared with studies on triacylglycerol emulsions. Several authors have reported that lipid oxidation is basically a surface phenomenon and extensive literature is available which indicates that antioxidants are very system dependant. Antioxidant studies with a range of model systems varying in complexity (other than in the "real food") can be misleading by over simplifying the interfacial interactions of multiple components (63).

The use of unsaturated fats or substrates could give representative results if the antioxidants are to be utilized in an oil, but unreliable results for measuring the early stages of lipid oxidation in a low fat food product. Hence standard vegetable oils could be used as suitable substrates to monitor the effectiveness of natural antioxidant extracts.

CHAPTER 3

SELECTION OF SUITABLE SUBSTRATE

3.1 INTRODUCTION

Lipid oxidation leading to rancidity is a decisive factor in determining the useful storage life of food products, even when their fat content is very low. Much research has been conducted to better understand the mechanism of oxidation of polyunsaturated lipids, antioxidants and the effects of decomposition products of lipid oxidation on the development of rancidity in foods (53).

To estimate the stability or susceptibility of a fat to oxidation, the sample is subjected to an accelerated oxidation test under standardized conditions and a suitable endpoint is chosen to determine signs of oxidative deterioration. Several parameters (e.g. temperature, metal catalysts, oxygen pressure, shaking) are manipulated to accelerate oxidation and the development of rancidity in vegetable oils and their emulsions. The induction period (I.P.) is measured as the time required to reach an endpoint of oxidation corresponding to either a level of detectable rancidity or a sudden change in the rate of oxidation (53).

Measurement of the I.P. under conditions described above is generally used as an index of antioxidant effectiveness. For practical purposes, however, predictions of oxidative stability in foods and oils based on measurements of I.P. should be related to measured product shelf life (53).

The induction period may be determined by storing the sample in an oven and periodically determining the oxidative state by an accepted method such as Peroxide Value, sensory evaluation, thiobarbituric acid test, active oxygen method, Swift test or Rancimat Test (55), and the modified method of Bishov and Henick by Ross and de Muelenaere (64). The Rancimat and the

Ross and de Muelenaere methods have been used for monitoring lipid oxidation in this investigation.

The Ross and de Muelenaere method is a static method involving monitoring the change in the nitrogen to oxygen ratio of the headspace over methyl linoleate stored at 50°C or 80°C. The Rancimat not only uses elevated temperature to accelerate oxidative rancidity but also is a dynamic method in that air is bubbled through the substrate. The method relies on the continuous monitoring of the electrical conductivity of the aqueous effluent to determine the induction period. A schematic diagram of a single Rancimat test setup is shown in Figure 3.1 (60).

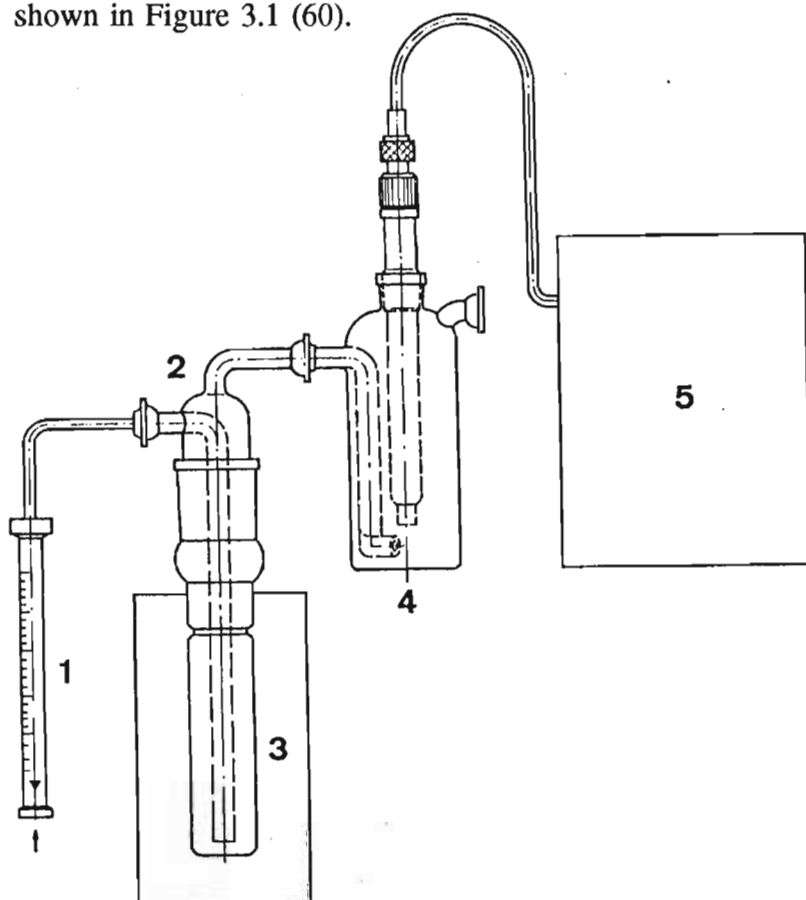


Figure 3.1 Schematic view of a single test setup in the 617 Rancimat:

1, flowmeter, air flow 10 l/hr; 2, reaction vessel for oil or fat samples; 3, aluminum heating block, temperature control to $\pm 0.1^\circ\text{C}$; 4, absorption vessel containing dist. water and double platinum foil electrode; 5, conductivity signal amplifier and 6-channel point recorder (60).

A graphical illustration of typical induction period determinations is recorded in Figure. 3.2. (60) The noted time value is determined by the intersection of the tangents to the different parts of the curve.

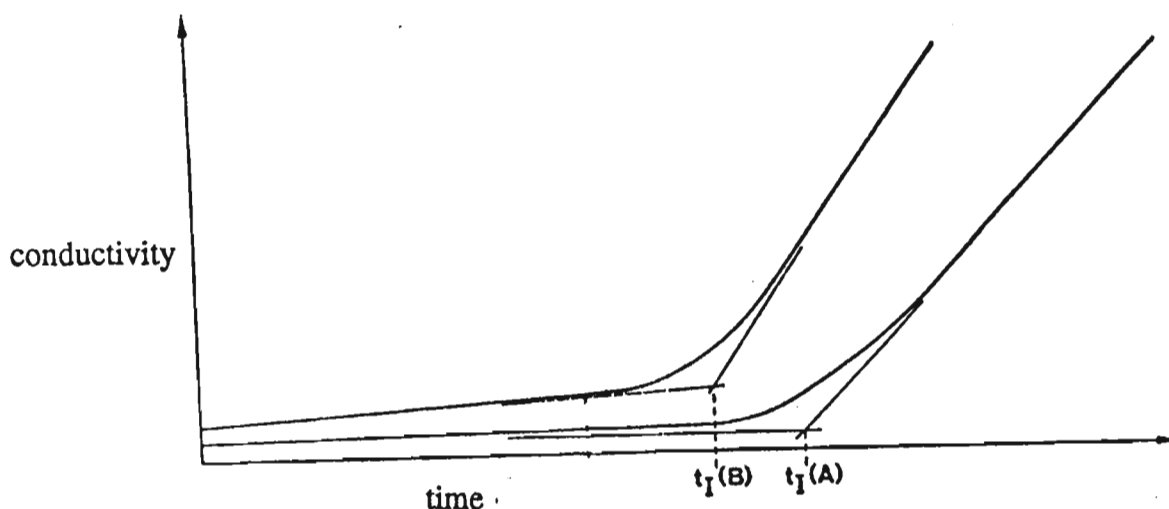


Figure 3.2 Graphic determination of the induction time (t_I), by the tangent method. A and B, typical conductivity curve (60).

A prerequisite for a suitable substrate is a low induction period such that the efficacy of the antioxidant can be determined in the substrate with respect to the induction period recorded. The ratio of the length (period) of the induction period of substrate with added antioxidant to the length (period) of the substrate without any antioxidant is used in conjunction with induction period of standard antioxidant to establish its activity.

Methyl esters of polyunsaturated fatty acids with 1,4 - pentadiene functional units, for example methyl linoleate, are particularly sensitive to oxidative reactions. Using methyl linoleate, oxidation can be initiated by the mechanisms of abstraction and "ene" addition (Figure. 3.3).

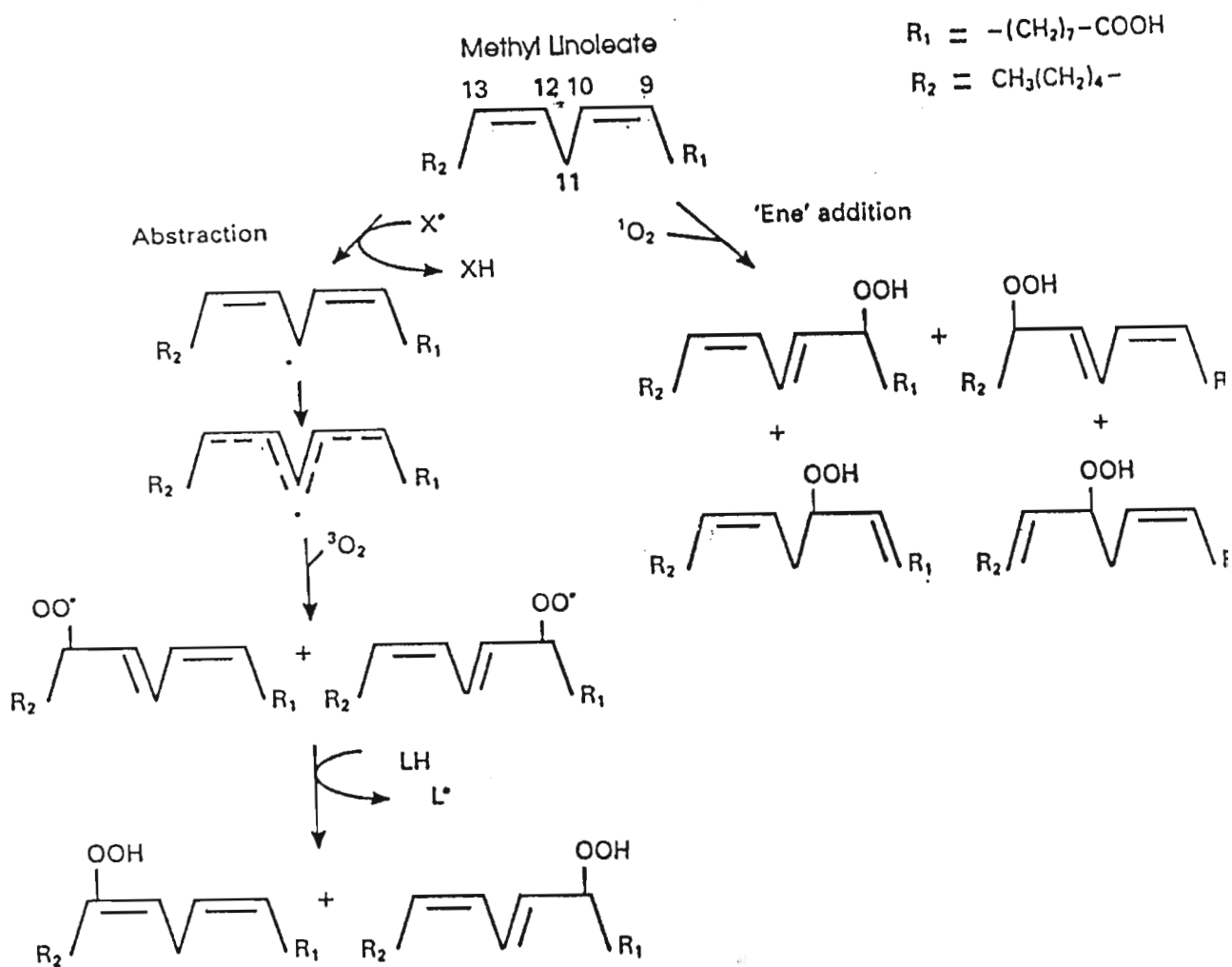


Figure 3.3 Initiation reactions for the oxidation of linoleate (54).

Abstraction is when an electron (or hydrogen atom) is removed from the fatty acid by reaction with an electrophilic species such as OH^\bullet or X^\bullet or

by interaction with high-energy radiation. The initial abstraction step yields a free radical (Initiation stage) which undergoes addition of $^3\text{O}_2$ (propagation stage) and then abstracts an electron from another biological compound. The resulting free radical ($\text{L}\cdot$) can be stabilized by resonance along the original pentadiene structure, and the fatty acid radical tends to undergo addition of $^3\text{O}_2$ when the unpaired electron is most "delocalised", that is at the C9- and C13-peroxyl radicals and then 9- and 13- hydroperoxides are formed (65).

The 'ene' addition reaction that can initiate lipid oxidation is caused by the highly electrophilic $^1\text{O}_2$, which will add directly to the double bond (high electron density). Thus a mixture of 9-, 10-, 12-, and 13-OOH isomers are produced by $^1\text{O}_2$ reaction with methyl linoleate (65). These products are unstable and additional secondary reactions take place which give rise to off-flavours and odours that are associated with oxidized foods or oxidative rancidity (65).

The rate of oxidation and the length of the induction period depends, among other things, on the fatty acid composition of the oil, the more allyl groups present the higher the oxidation rate and the shorter the induction period. In most substrates it is the unsaturated lipids such as oleate, linoleate, and linolenate, which are susceptible to autoxidation. The higher the degree of unsaturation, the greater the rate of deterioration of the fatty acids, because of the reactivity conferred by the double bonds. The methylene hydrogens of methylene-interrupted unsaturated systems are particularly prone to hydrogen abstraction. It has been found that linoleate reacts 30 to 40 times faster than oleate, and linolenate 80 to 100 times faster than oleate. The relative rates of autoxidation in a mixture of oleate, linoleate, and linolenate as reported in literature are 1 : 27 : 77 and 1 : 12 : 25 depending on the relative concentration of each fatty acid component (66). Hence two lipids with the same level of unsaturation or iodine number may differ in the reactivity of the unsaturated fatty acids towards oxidation. The different proportions of a more highly reactive fatty acid moiety present in one but not in the other necessitates the

calculation of a parameter called the Oxidative Susceptibility Value (OSV). This would account for the relative reactivities of the different fatty acids present in the oils (66). The OSV gives an indication of the relative reactivities of the different vegetable oils with respect to autoxidation (66). A method described by the South African Paint Research Institute to calculate the Iodine Values from the percentage of unsaturated fatty acids present in the oils was used and compared to Iodine Values obtained by chemical analysis. Similarly the Oxidative Susceptibility Value was calculated from the unsaturated fatty acids of the oils.

In this investigation the oxidation of methyl linoleate as well as several vegetable oils were monitored by the Ross and de Muelenaere method and the Rancimat test in order to select the most suitable substrate (with low induction period) to be used to evaluate antioxidant activity. The fatty acid profiles of the individual oils were determined by Gas Liquid Chromatographic analysis. Iodine values and Oxidative Susceptibility Values were calculated from the unsaturated fatty acids.

3.2 **MATERIALS AND METHODS**

3.2.1 **Materials**

3.2.1.1 **Methyl Esters**

Methyl Linoleate 99% pure, Sigma prod. no. L1876

Methyl Oleate 99% pure, Sigma prod. no. O4754

Methyl Stearate 99% pure, Sigma prod. no. S5376

Methyl Palmitate 99% pure, Sigma prod. no. P0750

3.2.1.2 **Vegetable Oils**

Refined Soyabean Oil ex CPC Tongaat Oil

Refined Sunflower Oil ex CPC Tongaat Oil

Refined Maize Oil ex Hudson and Knight

3.2.1.3 Reaction Vessel for Oxidation studies

100ml Serum-type reaction vials aluminium seals and butyl septa for reaction vials

3.2.1.4 Antioxidant stripping aids

Activated Charcoal Riedel-de Haen prod no. 18001

Kieselghur calcined Riedel-de Haen prod. no. 18514

3.2.2 Methods

3.2.2.1 Determination of induction period by the Ross and de Muelenaere method (64).

Gas chromatograph

A Varian model 3700 gas chromatograph equipped with a thermal conductivity detector and two columns in the Series Across-Detector (SAD) configuration was used. The G.S.C was set up with the two columns for the separation of firstly the air and carbon dioxide peaks after which the detector polarity was changed and subsequent separation of nitrogen and oxygen was achieved by the second column.

Columns

1st column: a 2m x 2mm (I.D) Porapak N 80/100 mesh

2nd column: a 3m x 2mm (I.D) Molecular Sieve 5 60/80 mesh

Temperature

Injector port at 130°C

1st column at 80°C

2nd column at room temperature (21-23°C)

Detector at 140°C

Detector

Thermal Conductivity Detector (TCD)

Carrier gas

Helium

Integrator

Varian 4400

Procedure

Two Whatman No.1 filter paper discs (45mm diameter) were placed onto the base of the serum-type reaction vials in order to allow the oil substrate to spread evenly as a thin layer. A .25g oil sample or a 1.5g of soya flour was massed accurately in to the vials. The vials were sealed with butyl septa and aluminium seals. An average of 6 vials per sample were prepared for oxidation studies with each vial containing approximately 250mg oil substrate. The sealed vials were placed in a constant temperature oven held at 50°C or 80°C. Sample vials were removed periodically from the oven, cooled for 30 minutes and subsequently 20µl of headspace was injected into the gas chromatograph. The ratios of the integrator counts for the nitrogen and oxygen peaks were calculated. These ratios were plotted against time. For each experiment the inflection of the curve, obtained by the tangent method, was noted as the induction period.

3.2.2.2 Determination of induction period via Rancimat method (60).

Apparatus

Metrohm Rancimat model 617 with cleaned glassware

Instrument temperature at 120°C

Chart speed at 1mm = 3 minutes

Airflow 20 l/hr.

Procedure

A four gram sample of oil was weighed accurately into the reaction vessel. The vessel was placed into the heating cell. 50ml deionised water was added to the absorption tube containing the electrodes. All parts were connected to the apparatus as per the operating instructions and the test was carried out for 16 hours. For soya samples, a 2.5g sample was dispersed into 4g of stripped sun flower oil substrate placed in the reaction vessel and oxidation carried out as per instructions. For the measurement of induction period the tangent method, as illustrated in Fig. 3.2 was used.

3.2.2.3 Kieselghur and activated charcoal treatment to remove natural antioxidants (67).

A method for removal of natural antioxidants was employed using kieselghur or either a single or double kieselghur treatment combined with activated charcoal.

Twenty grams of oil sample was diluted with 40ml diethyl ether. The mixture was treated with either 5g kieselghur or with 2g of a 1:1 mixture of kieselghur and activated charcoal by shaking in a mechanical

shaker for 30 minutes. The treated mixture was filtered and the ethereal filtrate evaporated to the original oil level. For the double treatment a further 2g of the kieselghur/activated charcoal mixture was added and the process repeated. The treated oil samples were stored at -16°C under nitrogen until required.

3.2.2.4 Transesterification of Vegetable Oils (68).

Reagents

Esterification mixture: 100 volumes methanol (A.R.) mix with 100 volumes chloroform (A.R.) and 1 volume concentrated sulphuric acid.

Apparatus for esterification

Bomb apparatus (as per Appendix 1) complete with heating block and thick walled test tubes.

Procedure

2 to 3 drops of sample was placed into the thick walled test tube. The tube was filled to the $\frac{3}{4}$ level with esterification mixture. The sample was well mixed with a glass rod and tested with blue litmus to ensure that the mixture was acidic. The tube was placed into the bomb apparatus and tightly sealed with an neoprene gasket. The esterification was carried out by heating the tube for 20 minutes at 180°C . The esterified mixture was cooled and subsequently washed 4 to 5 times with water. The chloroform layer was transferred to a clean dry test tube and gently heated. A stream of dry air was bubbled through the mixture to remove residual water and concentrate the solution. The sample of chloroform

containing methyl esters was used for fatty acid analysis by gas chromatography.

3.2.2.5 Determination of fatty acid composition by gas chromatography (68).

Gas chromatograph

A PYE UNICAM GCD chromatograph equipped with a Flame Ionisation Detector (FID)

Column

2m x2mm (ID) glass column packed with 5% DEGS-PS on 80/100 mesh chromosorb W-HP. A diethyleneglycol succinate treated with phosphoric acid.

Temperature

Injection port: 190°C

Oven temp: 185°C

FID temp:210°C

Carrier gas:Nitrogen at 30 ml/minute

Integrator:Varian model 4270

Procedure

A 2ul sample was injected into the gas chromatograph and peaks for the separated fatty acid esters recorded. The respective fatty acid esters were identified from standard fatty acid esters injected under identical conditions into the gas chromatograph. Integrator counts were used to obtain the percentage of each component in the mixture of the methyl esters.

3.2.2.6 Calculation of Iodine Values from Fatty Acid Composition (66).

The following equation was used by the South African Paint Research Institute to calculate Iodine Value (I.V) from the unsaturated fatty acids present in the oil.

$$\text{I.V.} = \text{MM I}_2 \times \left(\frac{\% \text{UFA}(1) \times N_1}{\text{MM UFA}(1)} + \frac{\% \text{UFA}(2) \times N_2}{\text{MM UFA}(2)} + \frac{\% \text{UFA}(n) \times N_n}{\text{MM UFA}_n} \right) \quad [1]$$

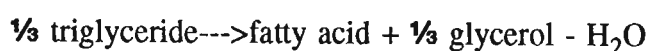
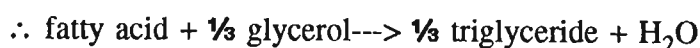
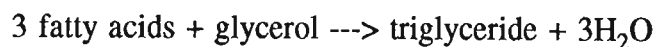
where MM I₂ = Molar Mass of Iodine

MM UFA = Molar Mass of Unsaturated Fatty Acid

N_n = No. of double bonds in any Unsaturated Fatty Acid UFA(n)

The above equation was corrected to convert the Iodine Value from a fatty acid to a triglyceride.

A conversion factor was obtained based on the following reactions:



$$\frac{\text{MM UFA}_n}{[\text{MM UFA}_n + \frac{1}{3} (\text{MM glycerol}) - \text{MM H}_2\text{O}]} \quad [2]$$

$$\frac{\text{MM UFA}_n}{[\text{MM UFA}_n + \frac{1}{3} (92.1) - 18.0]} \quad [3]$$

$$\frac{\text{MM UFA}_n}{[\text{MM UFA}_n + 13]} \quad [4]$$

By using the above factor, I.V. of fatty acids were converted to I.V. oil samples as follows

$$\text{I.V.} = \text{MM I}_2 \times \left(\frac{\% \text{UFA}(1) \times N_1}{\text{MM UFA}(1)+13} + \frac{\% \text{UFA}(2) \times N_2}{\text{MM UFA}(2)+13} + \frac{\% \text{UFA}(n) \times N_n}{\text{MM UFA}(n)+13} \right) \quad [5]$$

3.2.2.7 Calculation of Oxidative Susceptibility Value (66).

The OSV was calculated from the relative reactivity of the respective unsaturated fatty acids present in the oil samples using the following equation.

$$\text{OSV} = \frac{R_1 \% \text{UFA}(1)}{(\text{MM UFA}(1)+13)} + \frac{R_2 \% \text{UFA}(2)}{(\text{MM UFA}(2)+13)} + \frac{R_x \% \text{UFA}(x)}{(\text{MM UFA}(x)+13)} \quad [6]$$

R_1 the relative rate of oxidation of Oleic acid = 1

R_2 the relative rate of oxidation of Linoleic acid = 12

R_3 the relative rate of oxidation of Linolenic acid = 25

3.2.2.8 Determination of Iodine Value by chemical analysis (69).

Reagents

Carbon tetrachloride

Potassium Iodide 15% (300g/2l)

Sodium thiosulphate 0,1000N

Mercuric acetate solution. (25g/1l acetic acid)

Starch indicator (1g starch/250ml water. Boil and cool before use)

Wijs solution 0,2000N

Procedure

A 0.2g accurately weighed oil sample was dissolved in 15ml carbon tetrachloride in an iodine flask. 25ml Wijs solution and 10 ml mercuric acetate solution was added to the flask which was then stored in the dark for exactly 3 minutes. Thereafter a 100ml solution made up of 20ml of the 15% potassium iodide solution and 80ml water was added. The mixture was immediately titrated with sodium thiosulphate using starch indicator. Disappearance of the grey colour was taken as the endpoint. A blank determination was carried out at the

same time. Iodine value was calculated from the following equation.

$$\text{Iodine Value} = \frac{(\text{Vol blank} - \text{Vol sample}) \times 12,69 \times N}{\text{weight of oil sample}}$$

N = Normality of Sodium thiosulphate solution

$$12,69 = \frac{\text{Atomic mass of Iodine} \times 100}{1000}$$

3.3 RESULTS AND CALCULATIONS

3.3.1 Determination of Induction Period by Ross and de Muelenaere method

3.3.1.1 Induction period of methyl linoleate obtained by gas chromatography.

A typical chromatogram of headspace analysis of methyl linoleate before and after oxidation had taken place is shown in Figure 3.4

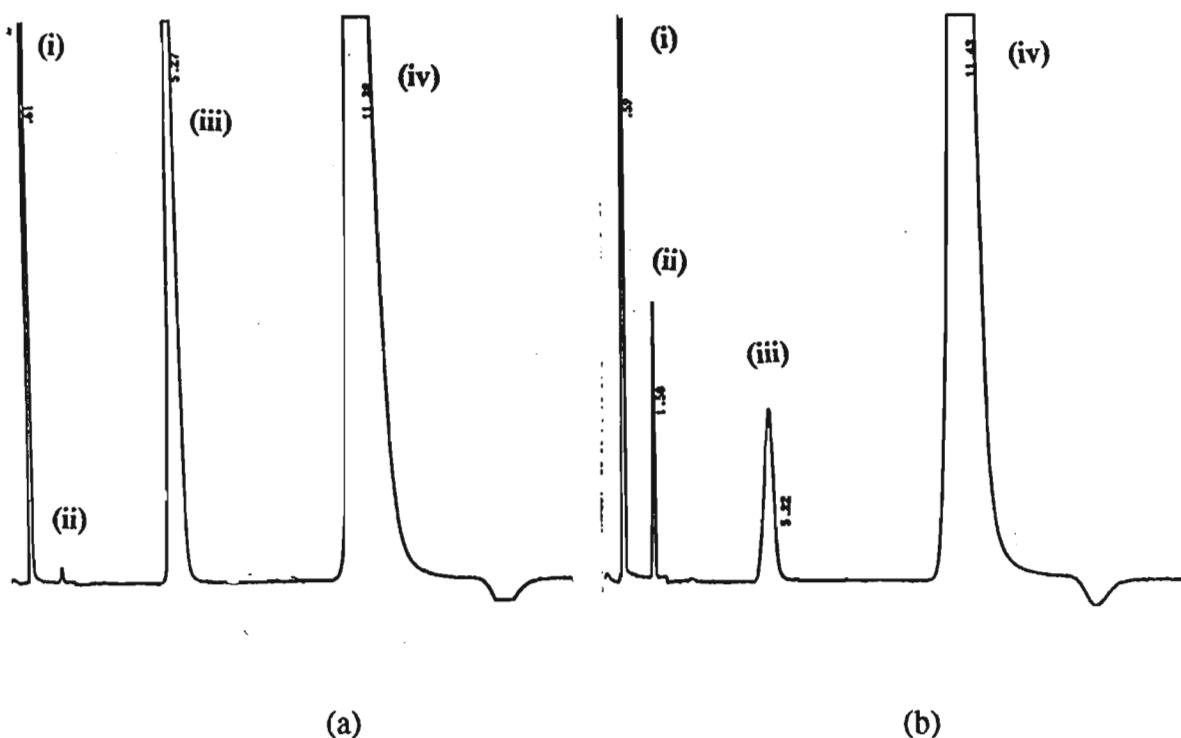


Figure 3.4 Headspace chromatograms of (a) unoxidised methyl linoleate and (b) oxidised methyl linoleate.

Peaks: (i) = composite air (ii) = carbon dioxide (iii) = oxygen (iv) = nitrogen.

Visual examination of the chromatograms show that the oxygen peak has decreased and the carbon dioxide peak has increased in the oxidised methyl linoleate sample. The carbon dioxide increased as a consequence of the oxidation process. The respective peak areas, as integrator counts, for nitrogen and oxygen were obtained from the printouts. These counts were used to calculate the nitrogen to oxygen ratios. A ratio of 3.87 and 35.2 for the unoxidised and oxidised methyl linoleate was obtained respectively.

3.3.1.2 Induction Period of methyl linoleate at 50°C

The oxidation of methyl linoleate was carried out several times to familiarize the investigator with the methodology and technique, as well as to establish that a 6 day 24 hour interval monitoring period was necessary to observe any meaningful oxidation change from the nitrogen to oxygen ratios recorded. The methyl linoleate substrates used were portions of a larger stock sample stored at 4-6°C

The following table indicates the N_2/O_2 ratios recorded for three independent runs of methyl linoleate conducted over a three month period and the induction periods recorded.

TABLE 3.1 Nitrogen to Oxygen ratios calculated for Methyl Linoleate at 50°C

Run	1	2	3
Hours	N_2/O_2	N_2/O_2	N_2/O_2
0	3.87	3.87	3.89
24	3.88	4.00	4.40
48	4.03	5.03	5.80
72	5.81	6.76	11.67
96	17.22	20.57	31.30
120	35.20	38.81	38.70
144	55.15	86.95	39.10
IP	78	75	62

A plot of N_2/O_2 against hours were drawn in Figures 3.5a, b, and c and the induction period of the respective runs was obtained by drawing tangents at the point of inflection.

The variation in induction period results from oxidation of methyl linoleate during subsequent sampling from stock sample. Due to this sensitivity to oxidation, vegetable oils were considered in place of methyl linoleate as suitable substrate.

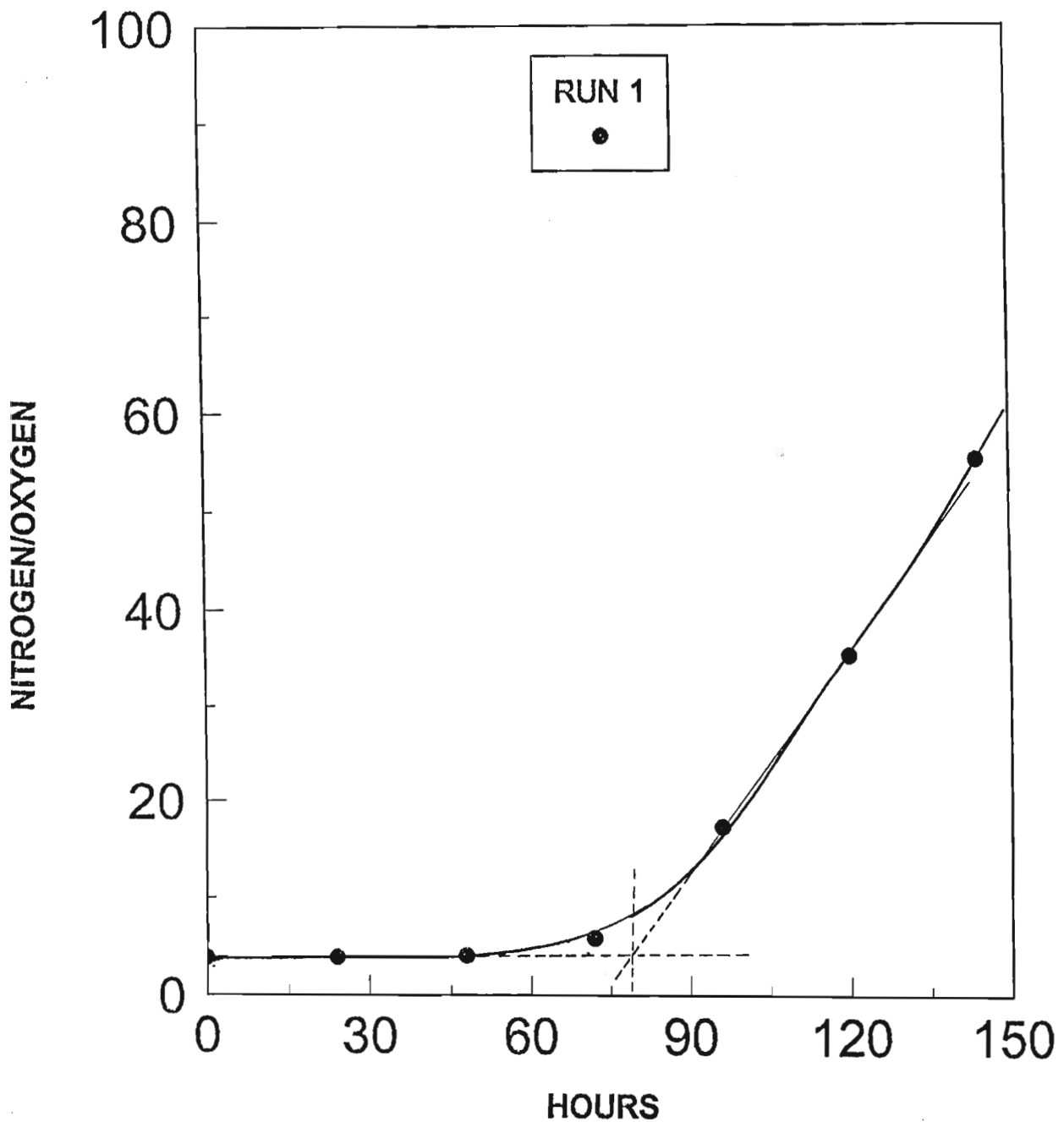


Figure 3.5a: Oxidation of methyl linoleate at 50°C (run 1).

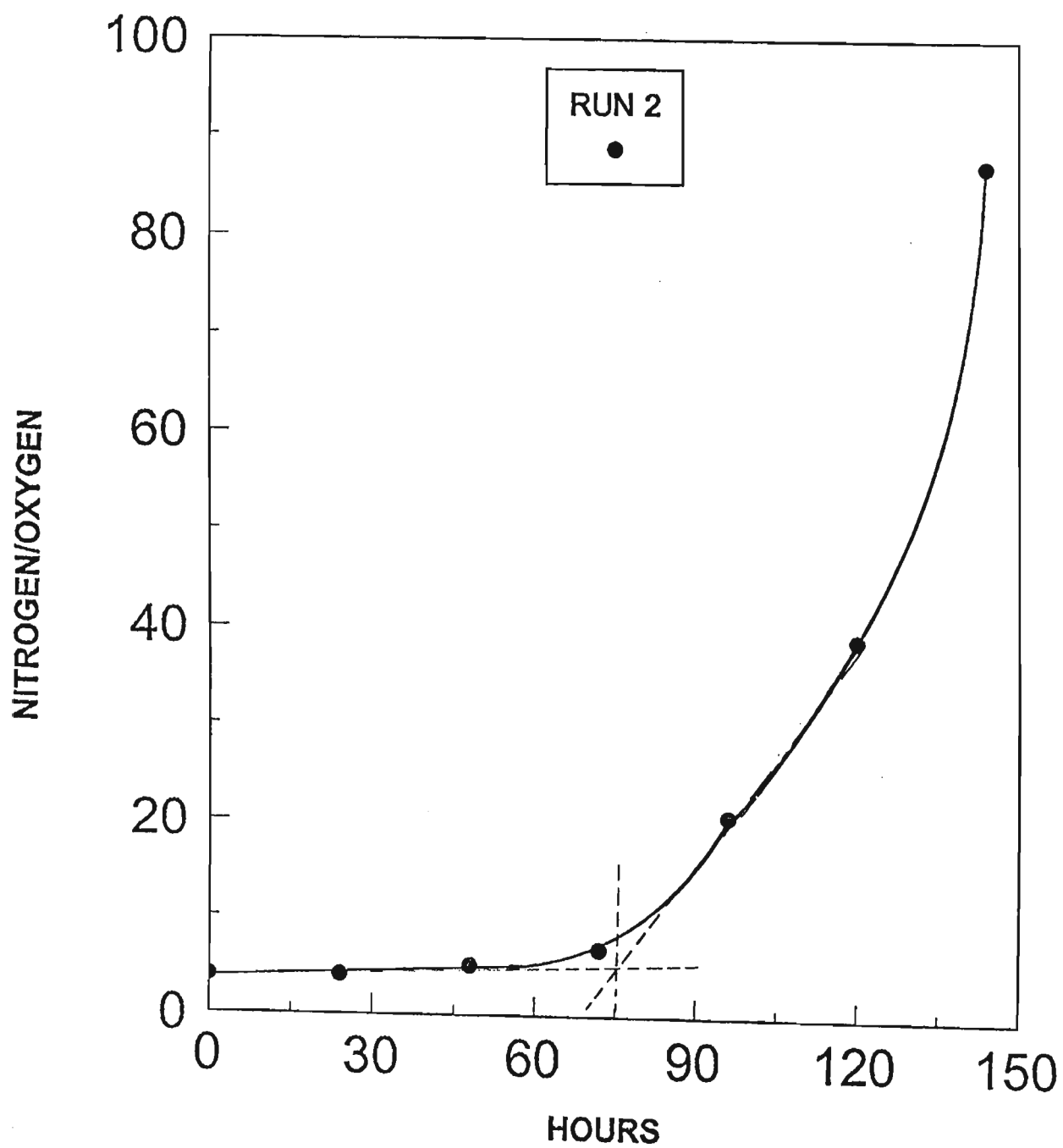


Figure 3.5b: Oxidation of methyl linoleate at 50°C (run 2).

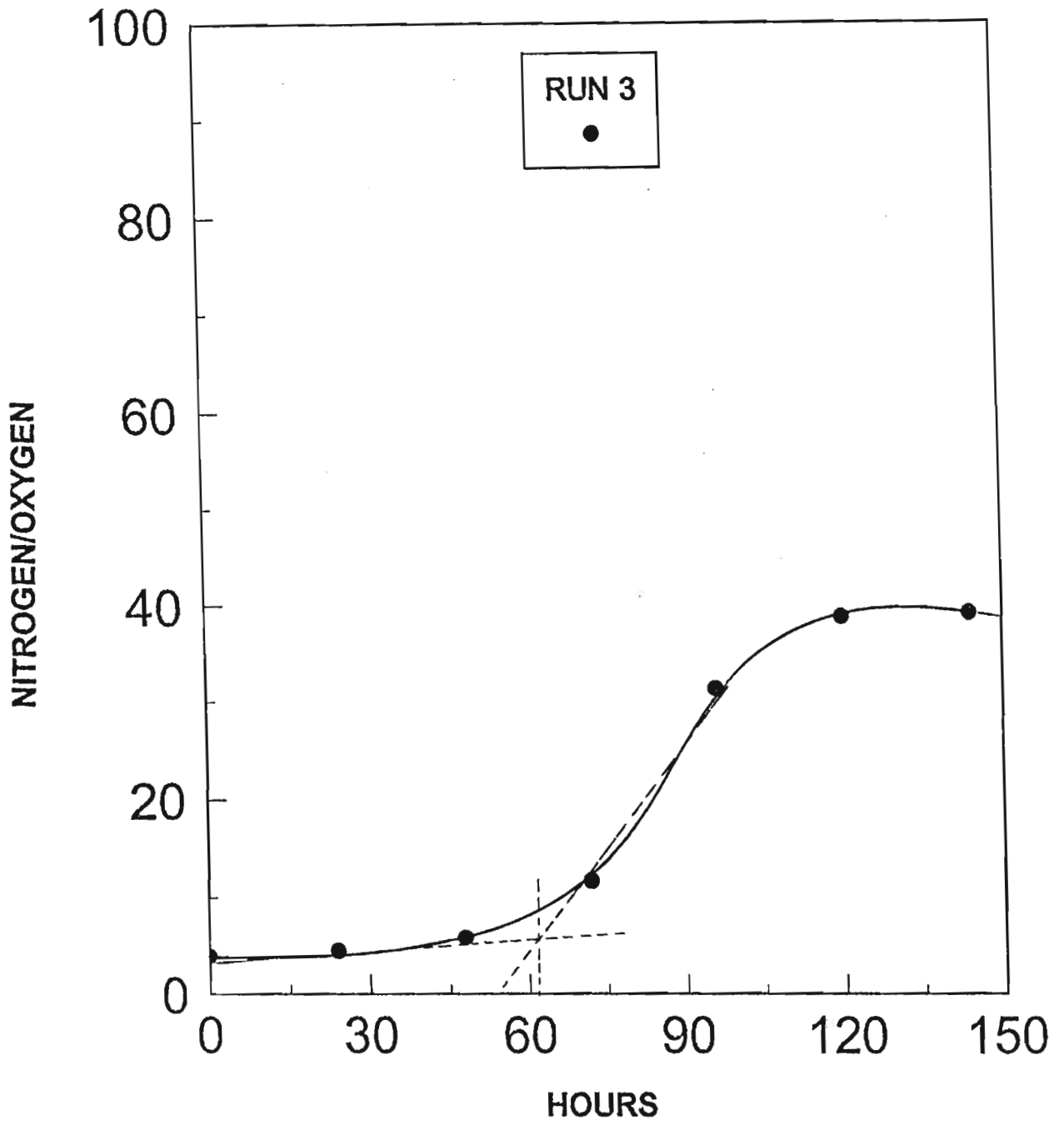


Figure 3.5c: Oxidation of methyl linoleate at 50°C (run 3).

3.3.1.3 Induction Period of vegetable oils at 50°C.

Refined, bleached and deodorized maize, soyabean and sunflower oils were monitored for oxidation and the following Table 3.2 lists the nitrogen to oxygen ratios of all three oils. The induction periods of the oils were calculated from the oxidation curves in Figures 3.6a, b and c.

Table 3.2 Nitrogen to Oxygen ratios calculated for maize, soyabean and sunflower oils

Samples	Maize	Soyabean	Sunflower
Hours	N_2/O_2	N_2/O_2	N_2/O_2
0	3.91	3.91	3.90
24	3.96	3.94	3.97
48	4.19	4.03	4.43
96	5.94	4.44	10.27
120	10.76	4.95	19.88
144	29.44	8.15	32.16

From the plots of N_2/O_2 ratios against hours the following induction periods of the oils were obtained.

Oil samples	Induction period
Maize	112.0 hrs
Soyabean	135.0 hrs
Sunflower	85.0 hrs

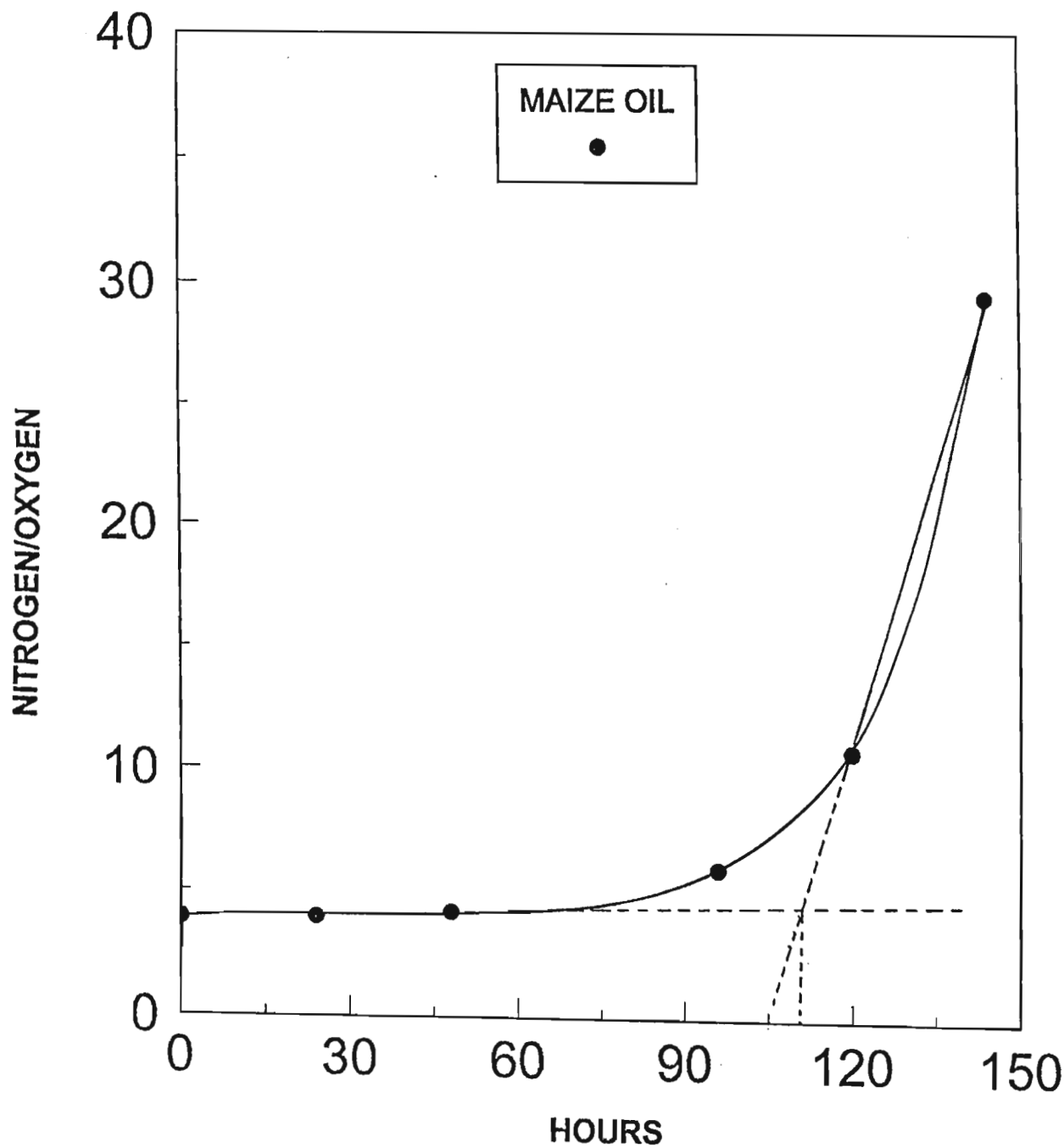


Figure 3.6a Induction Period of maize oil at 50°C

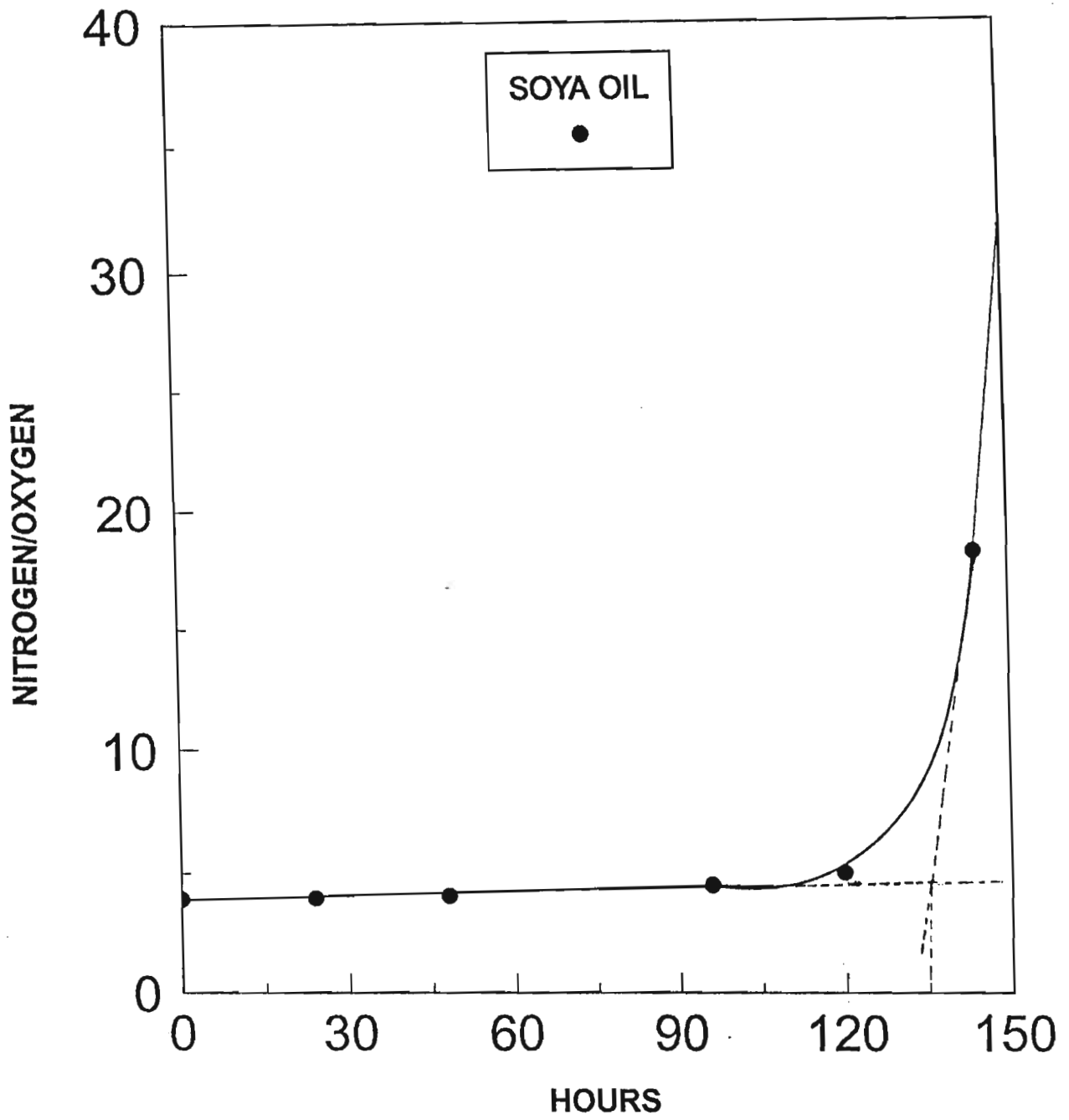


Figure 3.6b Induction Period of soyabean oil at 50°C

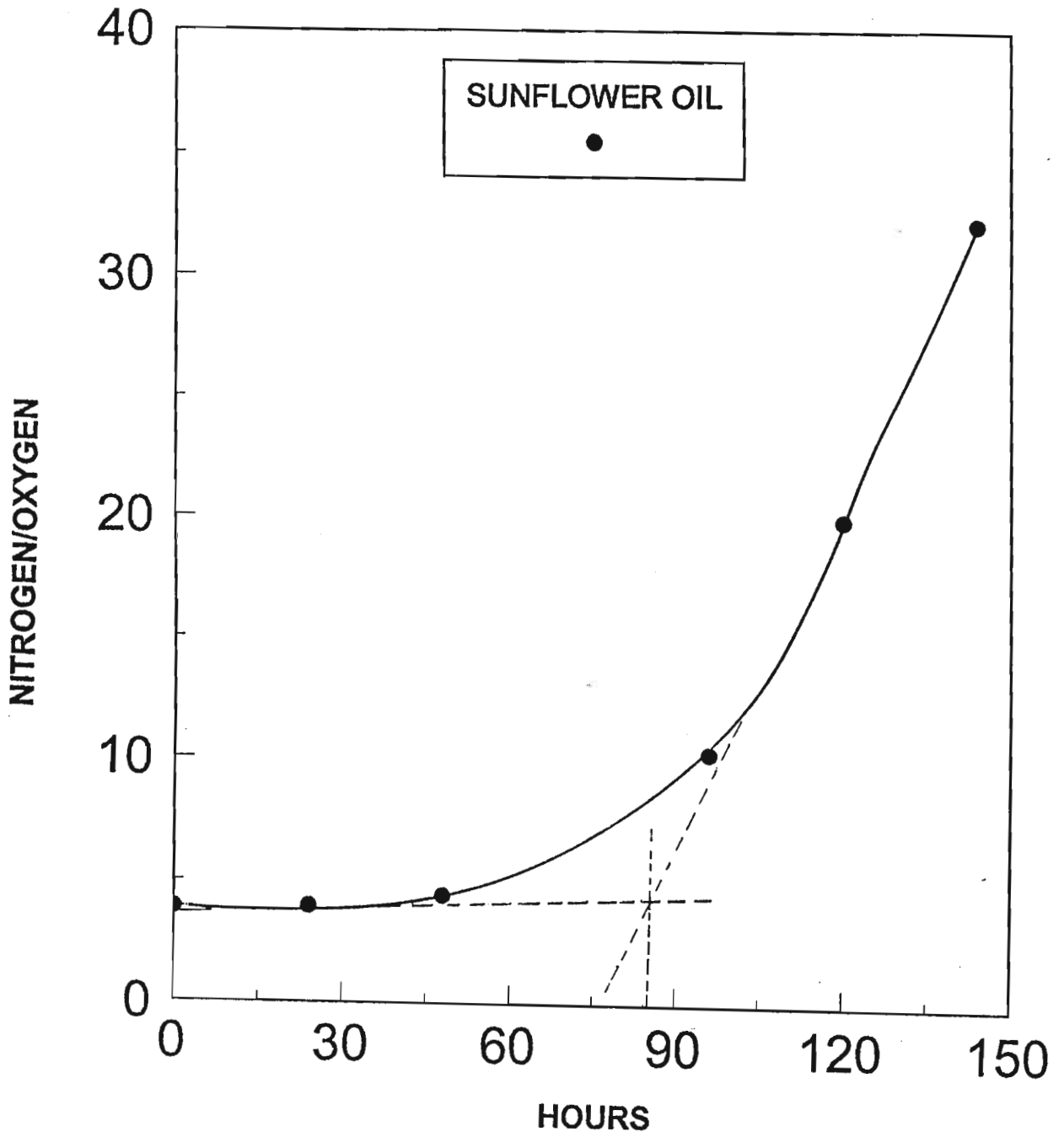


Figure 3.6c Induction Period of sunflower oil at 50°C

3.3.1.4 Induction Period of sunflower oils with and without kieselghur and charcoal treatment at 50°C.

Sunflower oil samples as is, treated with 5g kieselghur, treated with 2g of treatment mixture and a double treatment with 2g treatment mixture were monitored for oxidation and the following Table 3.3 lists the N₂/O₂ ratios. The induction period of the treated samples were calculated from the oxidation curves in Figures 3.7a, b, c and d.

Table 3.3 Nitrogen to Oxygen ratios calculated for the untreated and treated oil samples.

Samples	As is	5g kieselghur	2g mixture	2x2g mixture
Hours	N ₂ /O ₂	N ₂ /O ₂	N ₂ /O ₂	N ₂ /O ₂
0	3.86	3.87	3.87	3.88
24	3.90	3.89	3.91	14.96
48	4.33	4.58	10.34	47.81
72	4.98	8.88	23.48	88.23
96	9.79	14.36	41.05	132.7
120	18.37	26.78	61.86	194.17

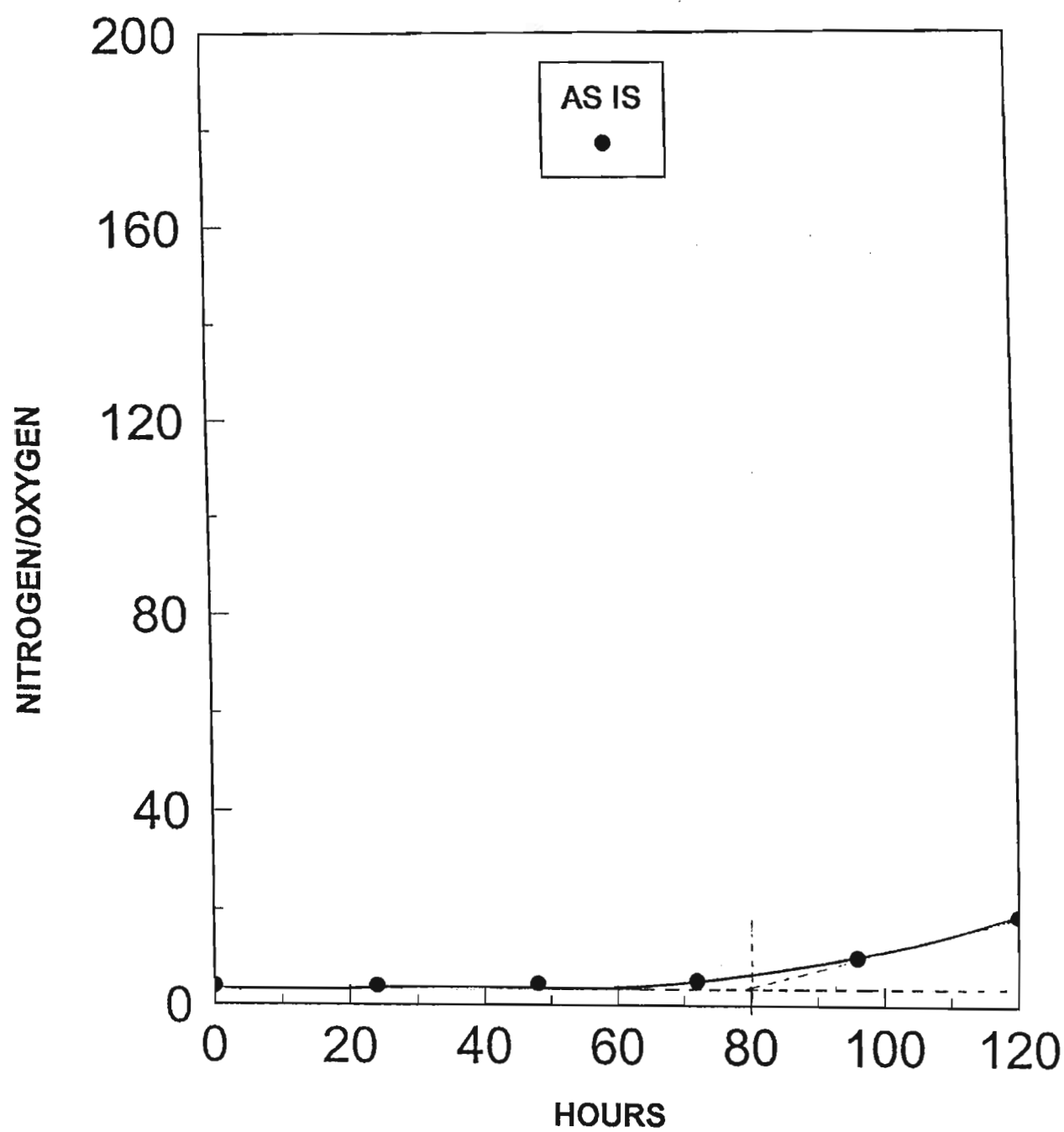


Figure 3.7a Induction Period of as is sunflower oil at 50°C.

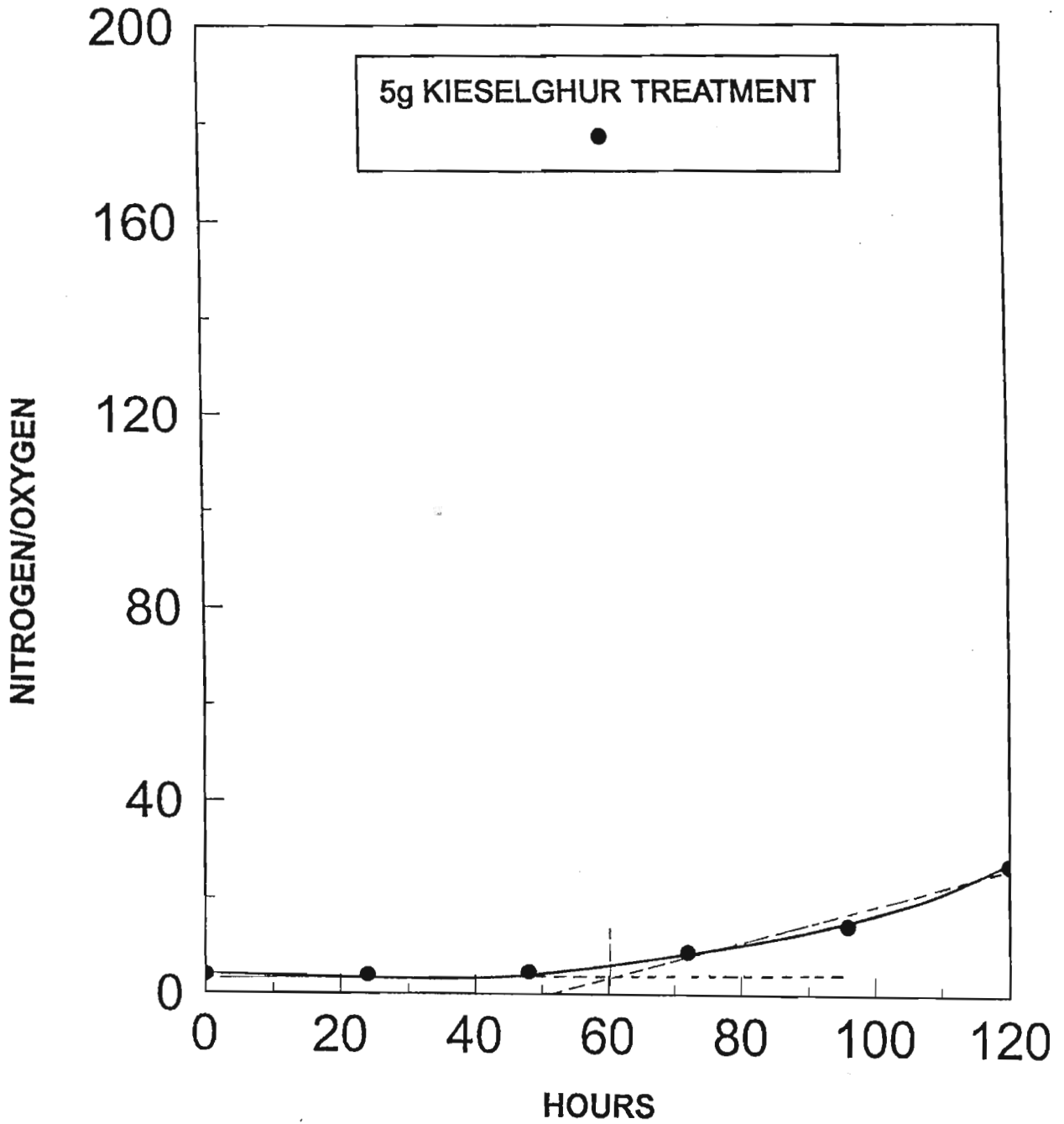


Figure 3.7b Induction Period of kieselghur treated sunflower oil at 50°C.

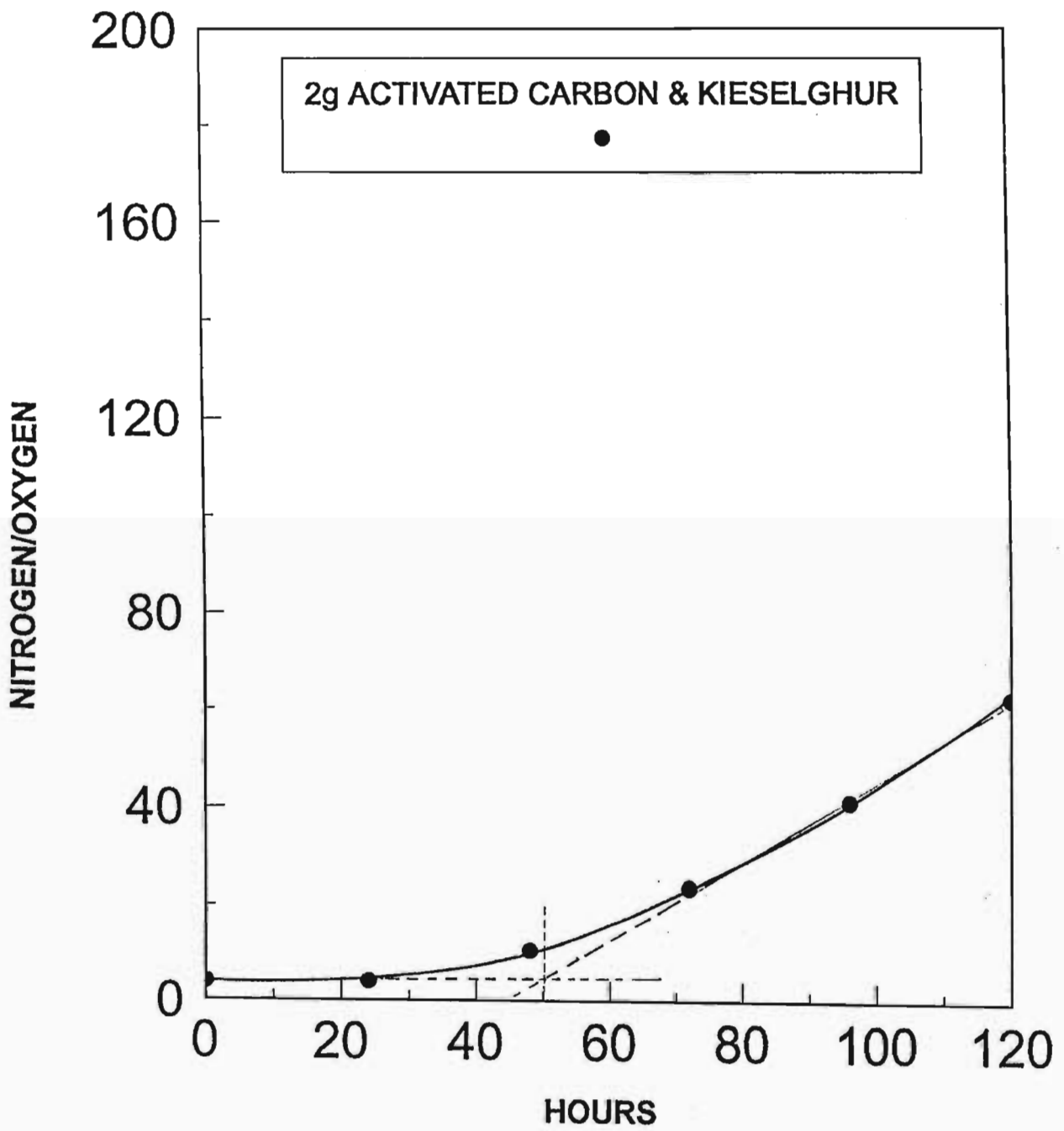


Figure 3.7c Induction Period of kieselghur and charcoal treated sunflower oil at 50°C.

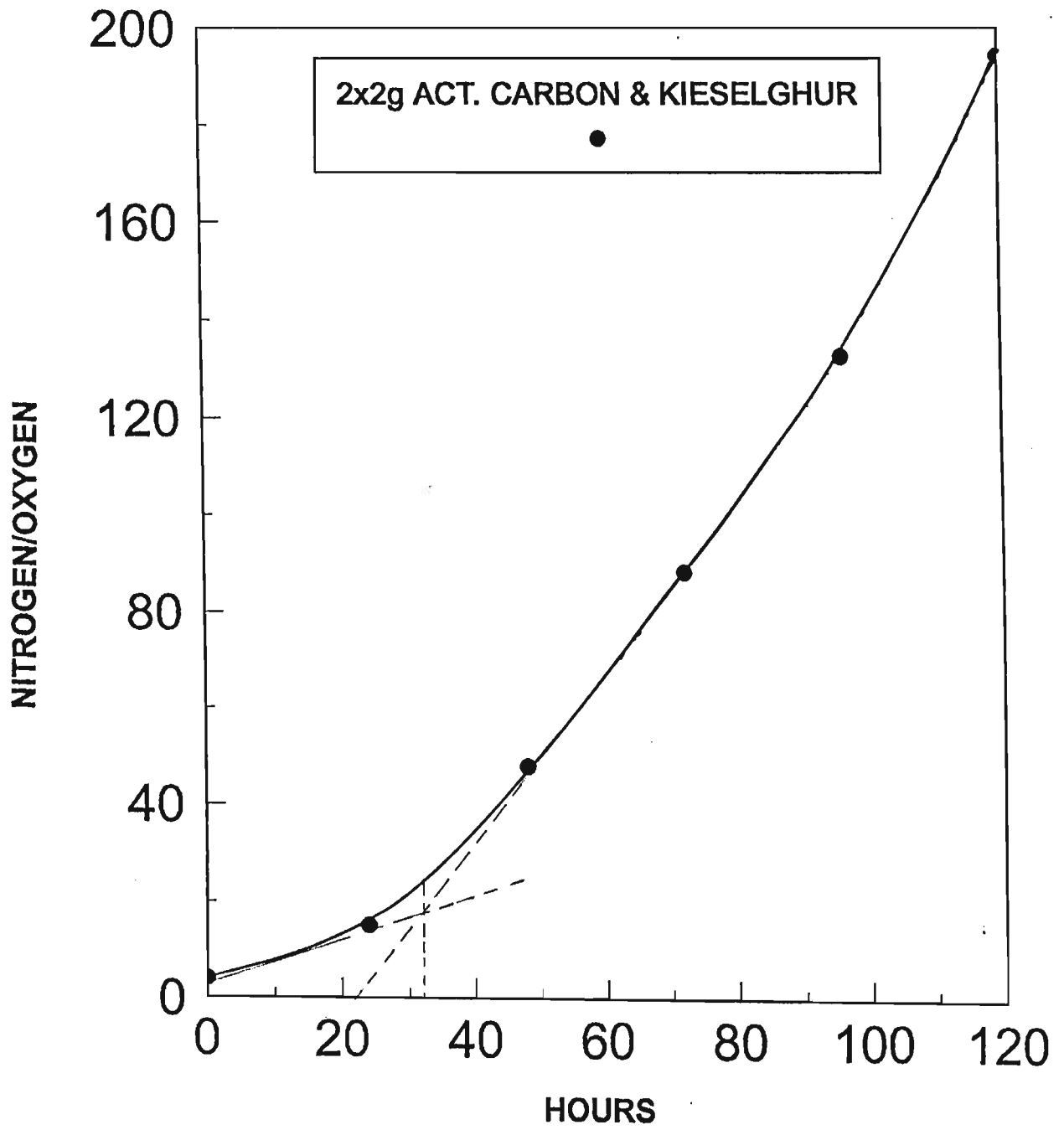


Figure 3.7d Induction Period of double kieselghur and charcoal treated sunflower oil at 50°C.

From the above plots of N_2/O_2 against hours, the following induction periods of the treated oils were calculated.

Oil samples	Induction periods
As is	84 hrs
5g kieselghur treatment	60 hrs
2g kieselghur/charcoal treatment	50 hrs
Double 2g kieselghur/charcoal treatment	33 hrs

3.3.2 Oxidation Studies at 80°C by Ross and de Muelenaere method

3.3.2.1 Induction period of Methyl Linoleate at 80°C

The oxidation of methyl linoleate was monitored at 80°C to establish the effect of increased temperature on the induction period. Table 3.4 lists the nitrogen to oxygen ratio monitored over the oxidation period and the induction period calculated from the oxidation curve shown in Figure 3.8

Table 3.4 Nitrogen to Oxygen ratio of methyl linoleate oxidised at 80°C.

Hours	N_2/O_2
0	3.89
1	4.37
2	5.16
3	6.41
4	16.08
5	34.88
6	91.94
7	110.73

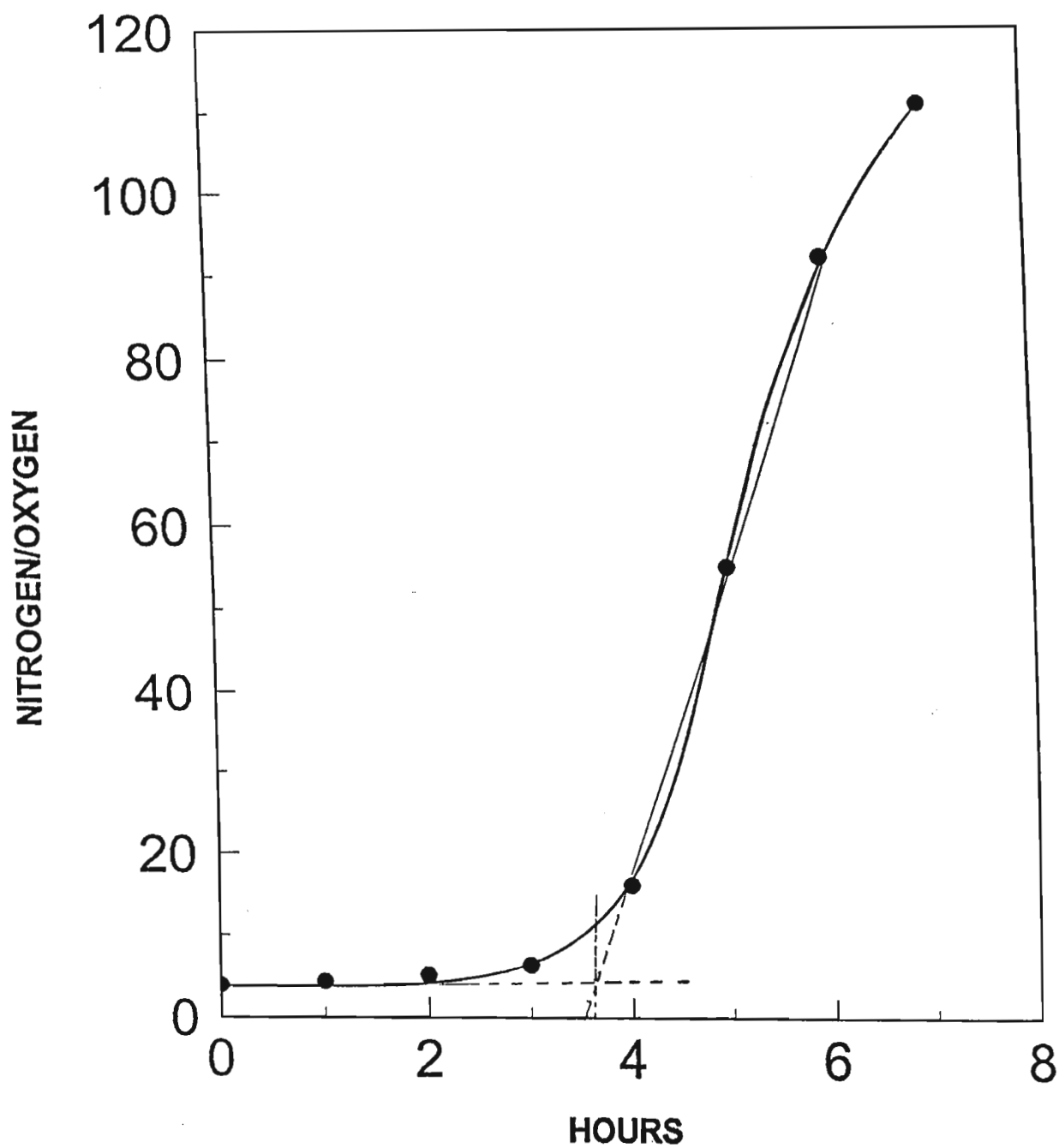


Figure 3.8 Induction Period of Methyl linoleate at 80°C

From the above curve the induction period of methyl linoleate was calculated to be 3,65 hrs.

3.3.2.2 Induction Period of Sunflower oil at 80°C

After several oxidation runs of sunflower oil samples to establish the critical hours at which changes of nitrogen to oxygen ratios occur, the following 3 runs of nitrogen to oxygen ratios listed in Table 3.5 were carried out at the same critical hours.

The induction periods of the 3 independent runs were calculated from the oxidation curves shown in Figures 3.9a, b and c.

Table 3.5 Nitrogen to Oxygen ratios calculated for Oxidation of Sunflower oils at 80°C.

Samples	Run 1	Run 2	Run 3
Hours	N ₂ /O ₂	N ₂ /O ₂	N ₂ /O ₂
0	3.90	3.89	3.87
24	3.98	3.93	3.88
48	4.14	4.27	3.97
50	4.32	4.61	4.04
51	4.71	4.82	4.51
52	5.27	5.44	4.98
53	6.33	6.76	5.76
54	7.19	7.48	6.93

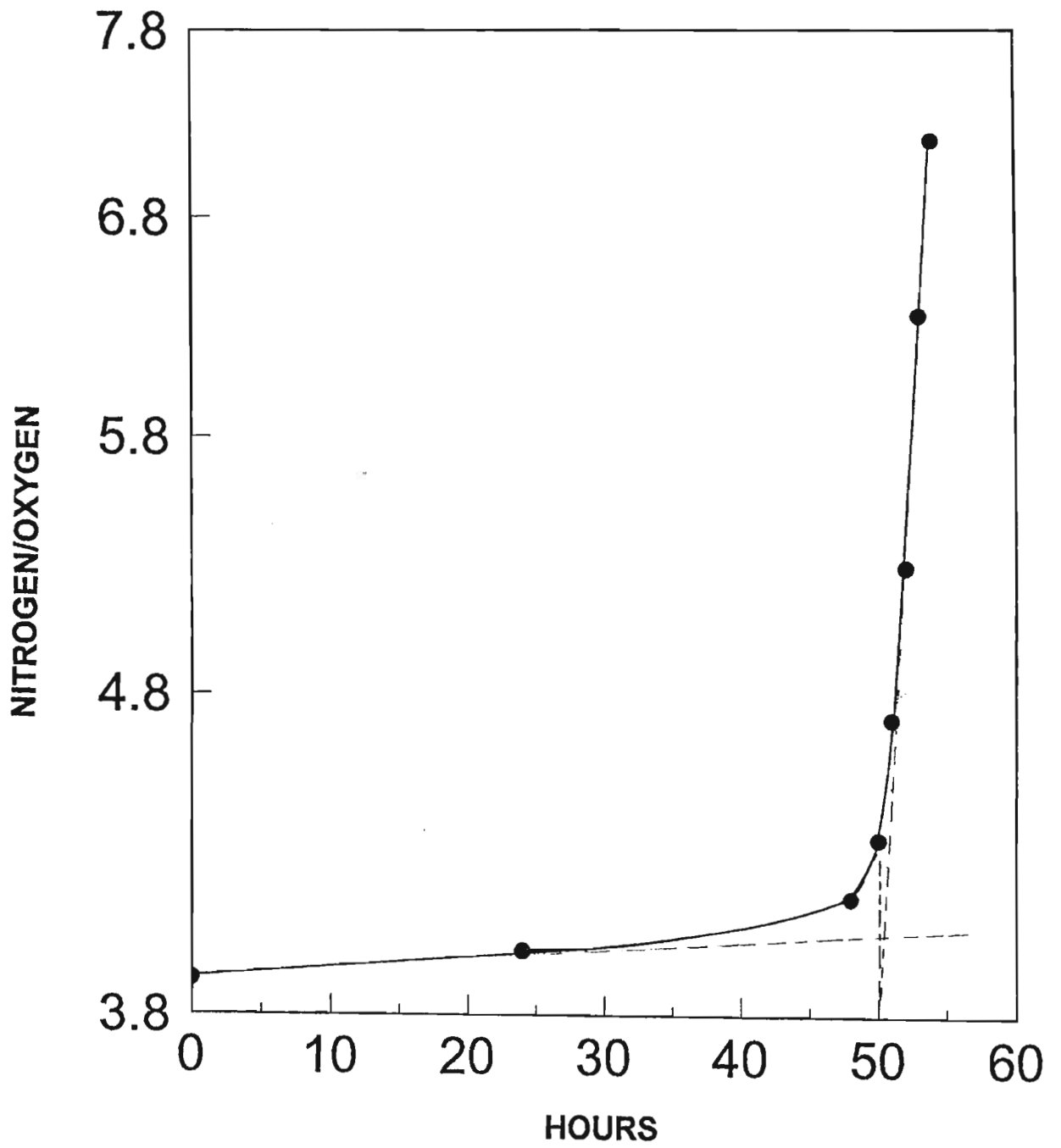


Figure 3.9a Oxidation of Sunflower oils at 80°C (Run 1).

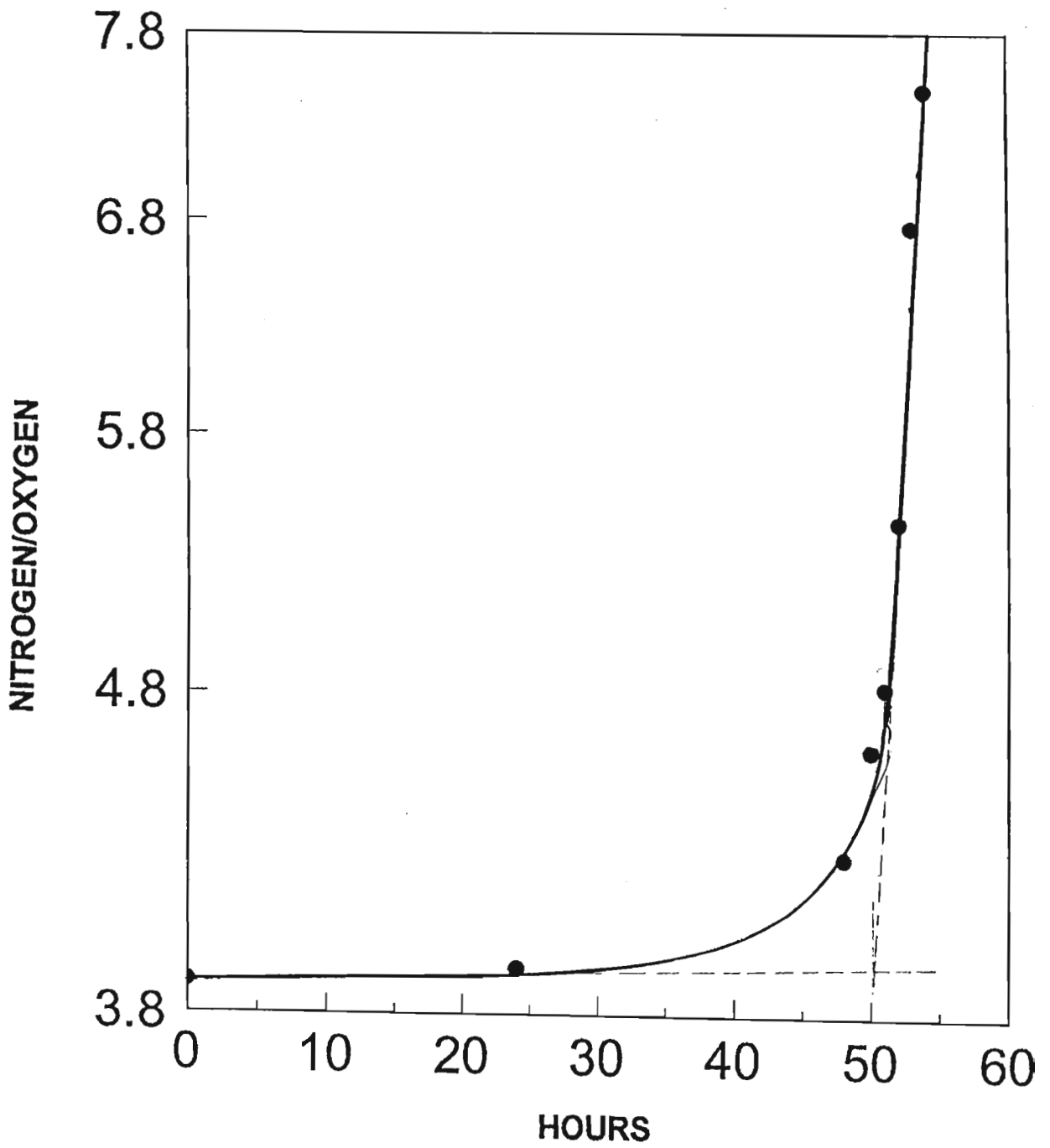


Figure 3.9b Oxidation of Sunflower oils at 80°C (Run 2).

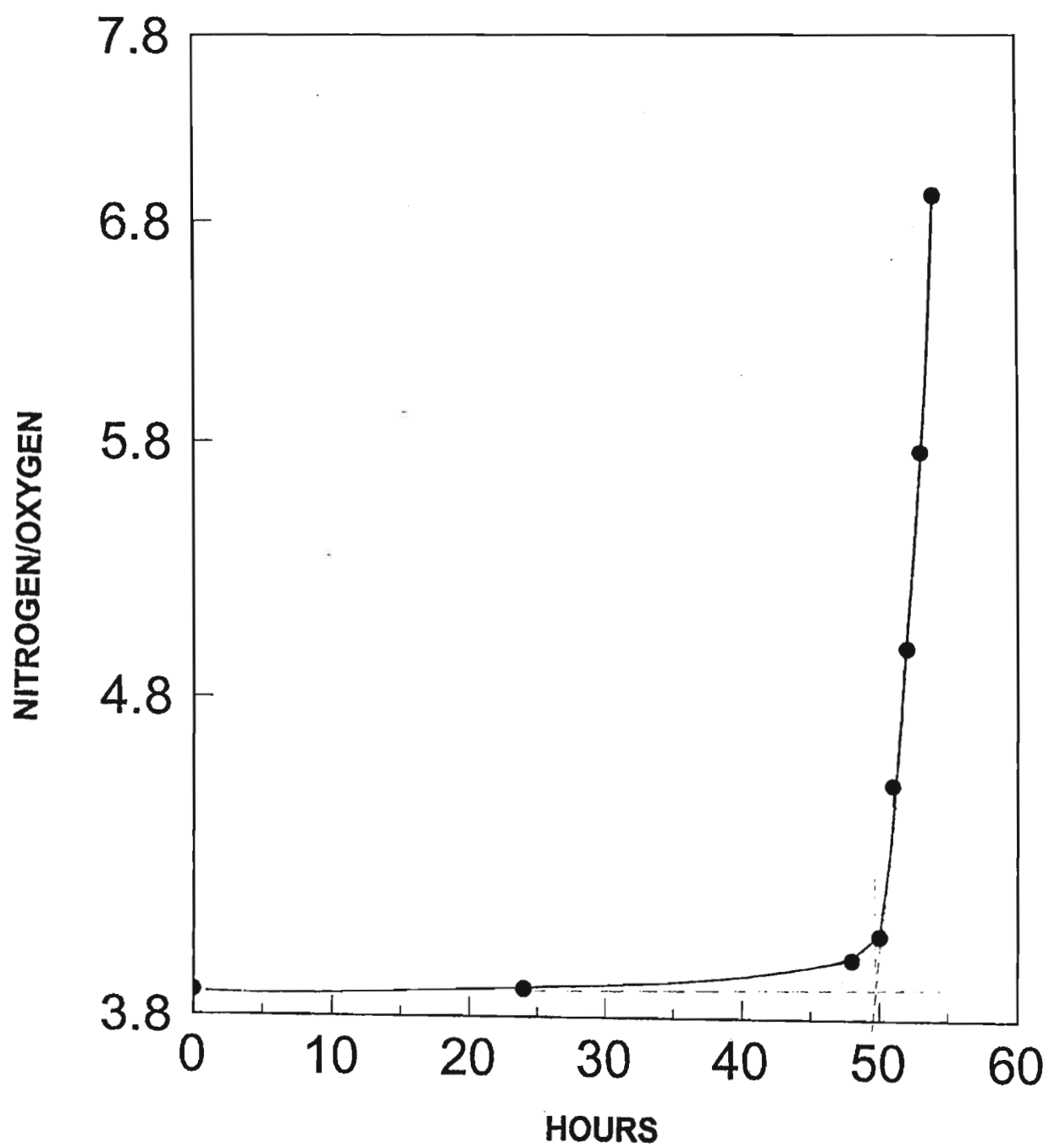


Figure 3.9c Oxidation of Sunflower oils at 80°C (Run 3).

Induction periods measured from the oxidation of sunflower oils
at 80°C

Oil samples	Induction periods
Run 1	50.0 hrs
Run 2	50.0 hrs
Run 3	49.2 hrs

3.3.2.3 Induction Period of sunflower oil stripped of natural antioxidants.

Sunflower oil was treated with a double 2g treatment mixture as reported in 3.3.1.4, to remove natural antioxidants, was monitored for oxidation. Table 3.6 lists the nitrogen to oxygen ratios calculated over the oxidation period.

Table 3.6 Nitrogen to Oxygen ratios calculated for sunflower oil stripped of natural antioxidants.

Sample	Stripped sunflower oil
Hours	N ₂ /O ₂
0	3.84
1	3.86
3	3.89
5	4.32
6	5.17
7	5.93
8	6.76
28	22.18

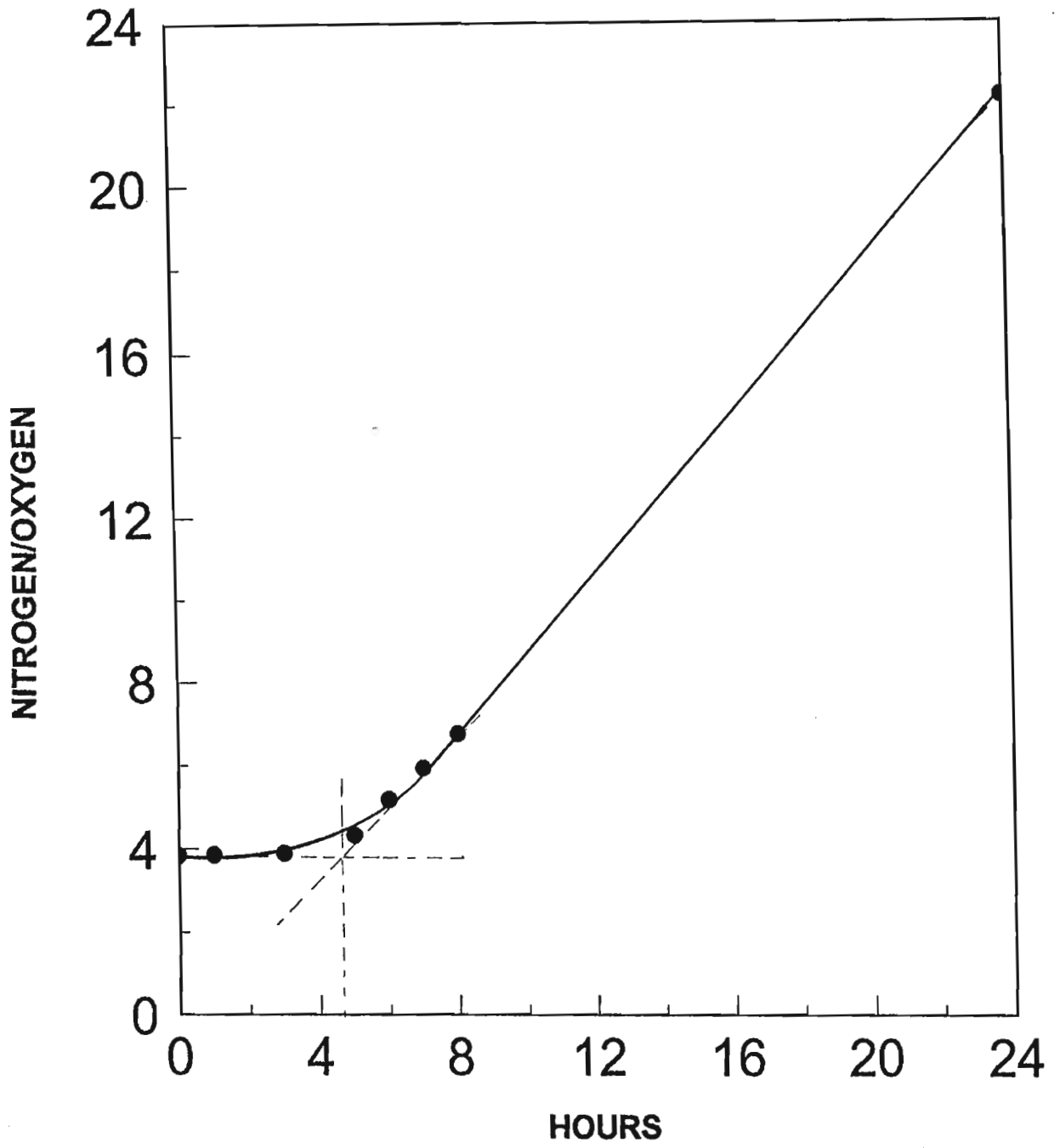


Figure 3.10 Induction Period of stripped sunflower oil at 80°C

From the above oxidation curve of stripped sunflower oil the induction period was calculated to be 4,5 hrs.

3.3.3 Oxidation Studies at 120°C by the Rancimat method

3.3.3.1 Induction Period of stripped and unstripped sunflower oils.

A sunflower oil sample stripped of natural antioxidants as well as an unstripped oil sample were oxidised in the Rancimat apparatus. From the inflection of the oxidation curve the induction period was determined. The following induction periods were recorded.

Oil samples	Induction period
Stripped sunflower oil	0.25 hrs
Unstripped sunflower oil	3.25 hrs

3.3.4 Summary of induction periods of oil samples at different oxidation temperatures.

Samples	Induction periods (hrs)		
	50°C**	80°C**	120°C*
Methyl Linoleate	71.7	3.5	-
Sunflower oil unstripped	84.0	49.7	3.25
Sunflower oil stripped	24.0	4.5	0.25

** = Ross and de Muelenaere method

* = Rancimat method

3.3.5 Fatty acid composition of Vegetable oils by Gas Chromatographic analysis.

3.3.5.1 Retention time of standard methyl esters.

The retention time of standard fatty acid methyl esters was determined by gas chromatography as described in 3.2.2.5.

Table 3.7 shows retention times of standard methyl esters obtained from chromatograms shown in Figure 3.11

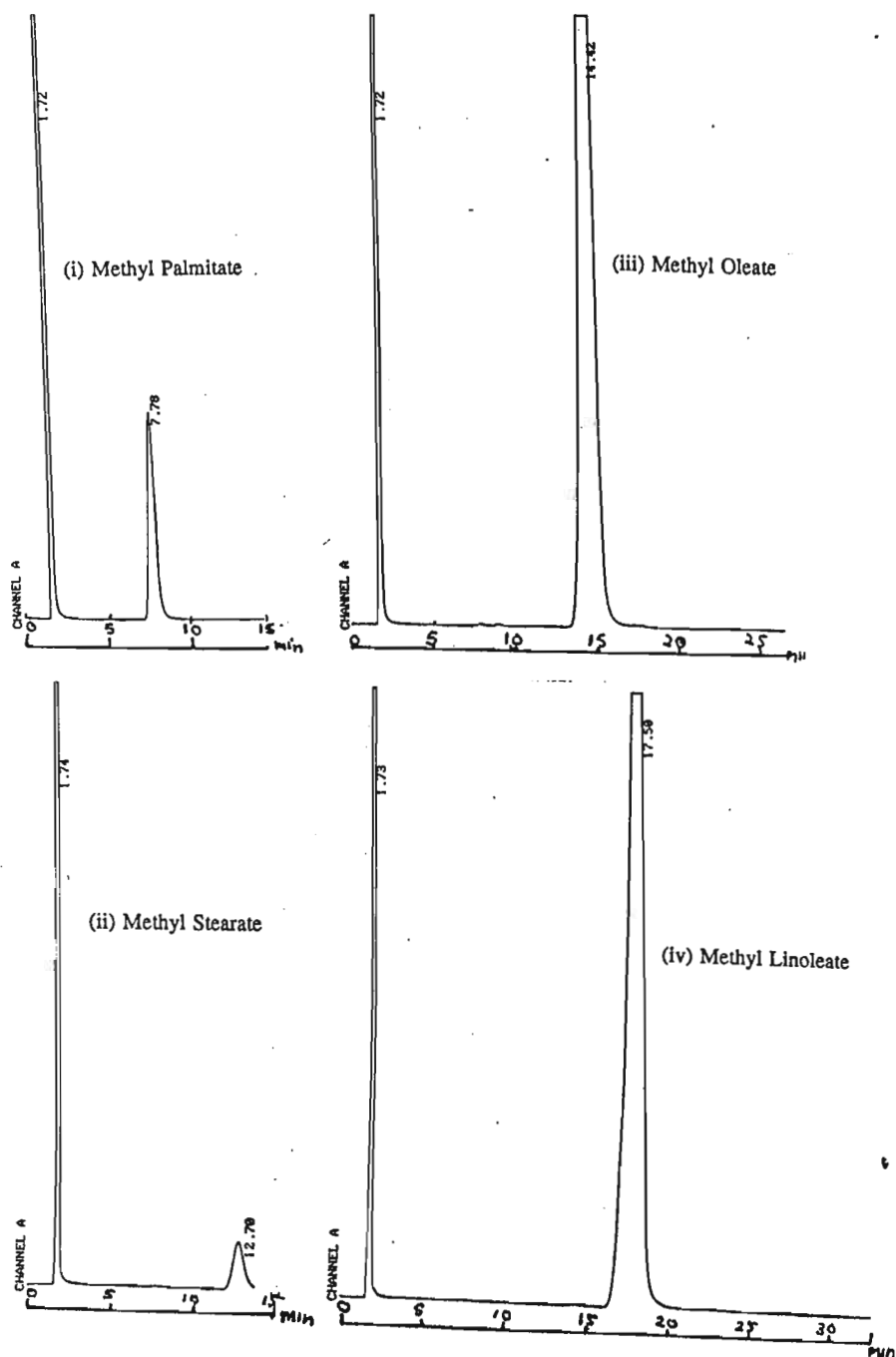


Figure 3.11 Chromatograms of standard methyl esters of Palmitic acid, Stearic acid, Oleic acid and Linoleic acid.

Table 3.7 Retention times of standard methyl esters

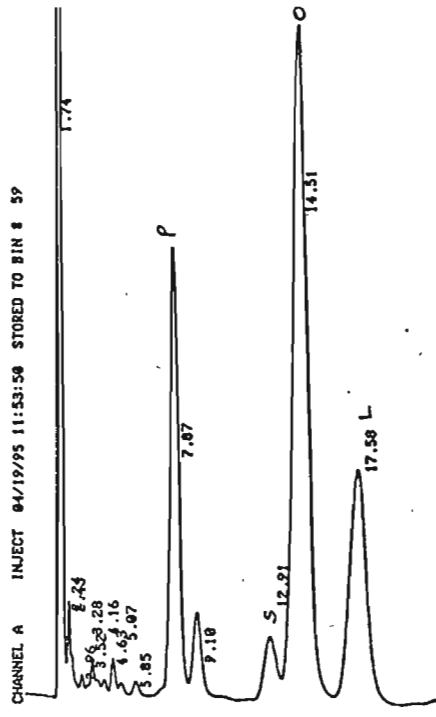
Methyl esters		Retention time
(i)	Methyl Palmitate	7.78min.
(ii)	Methyl Stearate	12.70min.
(iii)	Methyl Oleate	14.42min.
(iv)	Methyl Linoleate	17.50min.

3.3.5.2 Fatty acid composition of vegetable oils.

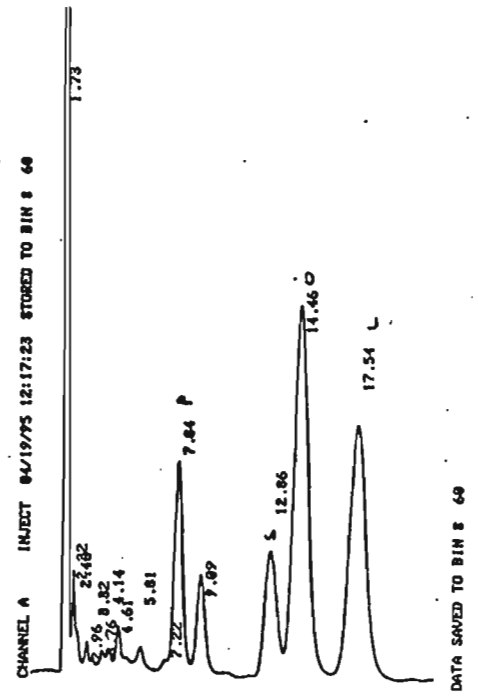
The fatty acid composition of maize, sunflower and soyabean oils were obtained from gas chromatographic analysis of methyl ester derivatives of the oils. The individual methyl esters were identified in the oil samples from the retention times of the standard methyl esters. The slight variation in retention times of the respective peaks results from the manual injection of the samples and time elapsed prior to start up of integrator.

Figure 3.12 shows the chromatograms of methyl ester derivatives of maize, sunflower and soyabean oils.

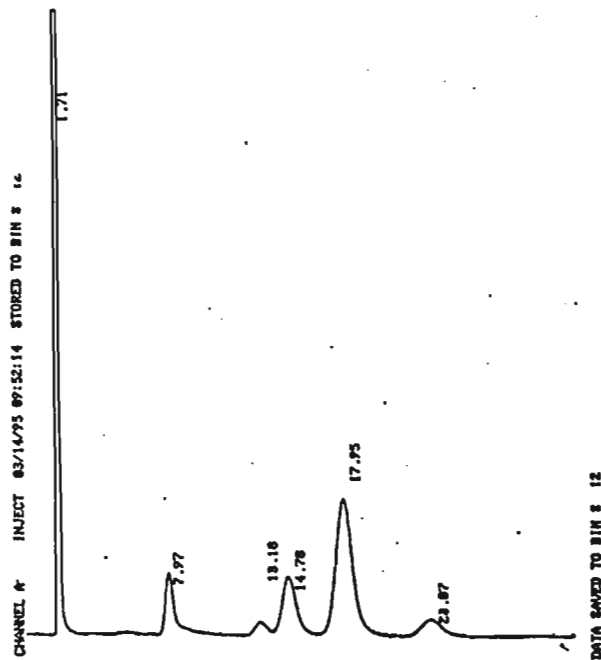
Table 3.8 shows the fatty acid distribution of maize, sunflower and soyabean oils taken from 3 independent gas chromatographic runs.



Maize



Sunflower



Soyabean

Figure 3.12 Chromatograms of Methyl Ester derivatives of Maize, Sunflower and Soyabean oils.

Table 3.8 Fatty acid composition of Soyabean, Maize and Sunflower oils.

Fatty acid	Palmitic	Stearic	Oleic	Linoleic	Linolenic
carbon no.	16:0	18:0	18:1	18:2	18:3
Soyabean oil					
	Saturated	Unsaturated			
Run 1	9.86	3.88	22.88	55.27	5.71
Run 2	10.12	4.12	23.29	54.59	5.96
Run 3	10.47	3.94	23.31	54.70	5.92
mean	10.15	3.98	23.16	54.85	5.86
s.d.	0.25	0.10	0.20	0.30	0.11
Maize oil					
Run 1	11.81	2.65	24.63	59.76	0.50
Run 2	12.16	2.70	24.81	60.41	0.50
Run 3	11.72	2.55	25.16	59.83	0.50
mean	11.90	2.63	24.86	60.00	0.50
s.d.	0.19	0.06	0.22	0.29	0.00
Sunflower oil					
Run 1	6.94	6.08	18.37	69.60	-
Run 2	6.53	6.31	18.12	69.80	-
Run 3	6.58	6.48	17.80	70.30	-
mean	6.68	6.29	18.10	69.90	-
s.d.	0.18	0.16	0.23	0.29	-

From the above Table 3.8 the total unsaturated fatty acids for the respective oils were obtained

% Unsaturated fatty acid

Soyabean oil	83.87
Maize oil	85.36
Sunflower oil	88.00

3.3.5.3 Determination of Iodine Value by chemical analysis and calculation of such from unsaturated fatty acids.

Iodine Values which give an indication of the degree of unsaturation were determined using method described in 3.2.2.8

and compared to Iodine Values obtained from calculations based on the unsaturated fatty acids, indicated by the chromatographic data.

From the unsaturated fatty acids listed in Table 3.7 and the equation in 3.2.2.6 for calculation of Iodine Value, the following Iodine Values, together with those obtained by chemical analysis, are listed in Table 3.9

Table 3.9 Iodine Values of vegetable oils.

Samples	Iodine Values	
	by Analysis	by Calculation
Soyabean oil	133	130
Maize oil	128	127
Sunflower oil	135	137

3.3.5.4 Determination of Oxidative Susceptibility Values of vegetable oils.

The OSV was calculated from the unsaturated fatty acids of the vegetable oils using the equation described in 3.2.2.7. The OSV values of the different oil samples are listed in Table 3.10 below.

Table 3.10 Oxidative Susceptibility Value of vegetable oils.

Samples	OSV
Soyabean oil	2.83
Maize oil	2.58
Sunflower oil	2.92

3.4 **DISCUSSION.**

The main aim of the work covered in this chapter was to select a suitable substrate for monitoring lipid oxidation. Methyl linoleate has been used by several researchers, this investigation confirmed that methyl linoleate is very sensitive to oxidation. This was evident from the reduced induction periods recorded from oxidation studies of methyl linoleate (section 3.3.1.2) over a 3 month period, using portions of the same sample stored at 4-6°C. This poor shelflife (considering that this project would extend to more than 3 months) as well as the relatively high cost and quantities required for investigation, prompted a further investigation into the use of other suitable substrates for measuring lipid oxidation.

One of the prerequisites for a suitable substrate for monitoring antioxidant activity is a short induction period such that upon addition of antioxidants the extended induction period is easily measured. From oxidation studies of soyabean, maize and sunflower oils by the two methods employed, sunflower oil was demonstrated to be most unstable due to the short induction period recorded, not only due to the presence of highly unsaturated fatty acids as indicated by fatty acid composition but also the slightly higher iodine value obtained by analysis and from calculations. The slightly higher Oxidative Susceptibility Value calculated for sunflower oil could possibly substantiate it as a suitable substrate for this investigation. The oxidation studies carried out at various temperatures stems from a need to increase the oxidation rate which reduced experimental time such that more exercises could be executed. The experiments clearly indicate that as temperature increases the oxidation rate increases and there is a marked decrease in induction periods. Most accelerated methods used for monitoring lipid oxidation involves the use of elevated temperatures as it is known that the rate of the oxidation reaction is exponentially related to temperature (70).

Stripping oils of their natural antioxidants, which would otherwise interfere with antioxidant studies of the Maillard reaction products, was

essential to the success of further studies. Based on the results reported in section 3.3.1.4 a double treatment of 2g each of kieselghur and activated charcoal was selected.

CHAPTER 4

EVALUATION OF STORAGE STABILITY OF SUBSTRATE.

4.1 INTRODUCTION

The flavours of plants and fish oils, due to their high levels of unsaturation, deteriorate very rapidly if oxidation is allowed to take place. Lipid oxidation often gives rise to "fishy" flavours and aromas which limits acceptability. Refining procedures for vegetable and seed oils are partly designed to remove chlorophyll which can also cause flavour deterioration as a result of initiating photo-oxidation reactions (65).

Methods used to stabilize foods against lipid oxidation are (11):

- 1) Storage at low temperatures.
- 2) Use of fats and oils that contain low levels of oxidation promoters, that is, transition metals.
- 3) Use of ingredients that are natural and rich in antioxidant activity.
- 4) Use of partly stabilised oils containing less unsaturated fatty acids.
- 5) Elimination of oxygen by inclusion of an oxygen scavenger in packaging material impermeable to oxygen.

Nitrogen flushing of containers and packing under vacuum are used to limit the amount of oxygen available for oxidative reactions (71). During the early 1960's, the use of nitrogen to protect a product against oxidative rancidity during manufacture was sparsely practised. Presently nitrogen flushing to protect the oil from oxidative deterioration is in general use (71). Studies have shown without any doubt the beneficial impact of nitrogen flushing in maintaining oil quality during storage. The results of the effects of nitrogen and air storage conditions indicated that even low levels of oxidation products that form while oil is held in bulk storage have a deleterious effect on shelf life. Thus the exclusion of oxygen during storage is highly desirable and is a practical method for preventing oil quality

deterioration (72). This exclusion of oxygen is generally achieved by using one of the following processes:

- a) Removing air and replacing it with nitrogen, carbon dioxide or any inert gas.
- b) Placing the product in a gas impermeable package and removing the air to create a vacuum.
- c) Sparging and nitrogen blanketing techniques to protect the oil during storage.

The sparging technique represents a practical method for protecting oils from oxidative deterioration during shipment from refinery to destination (72). The principle involved is saturation of the oil with nitrogen. Effusing gas sweeps out the headspace and thus removes most of the air and oxygen from the vessel. The gradual flushing action of sparge gas reduces the reabsorption rate of oxygen due to the pressure differential between the liquid and the headspace above it (71).

An increase in free fatty acids results from the breakdown of lipids and is manifested by off-flavours such as soapiness, increased acidity, and increased oxidation of fatty acids (73).

The measurement of peroxide value is an indicator of oxidative deterioration. Though peroxides and hydroperoxides of oil and fats are tasteless, their presence is an indicator that deterioration is occurring and their breakdown products do yield off-flavours (74).

Iodine Value is used as a measure of overall unsaturation to characterise oils and fats. Oxidative rancidity affects first the more unsaturated fatty acids, and as it progresses, polyunsaturated fatty acids polymerise or breakdown to smaller molecules with fewer double bonds. The Iodine Value decreases as the ratio of unsaturated fatty acids to total fatty acids is also decreased (74).

The results recorded in section 3.3.4 on summary of induction periods shows that methyl linoleate has lower induction period than sunflower oil stripped. This fulfils the prerequisite for a suitable substrate but due to its high cost and the quantity required for subsequent evaluation of Maillard reaction antioxidants, sunflower oil stripped was favoured as a suitable substrate. Furthermore, as cited in literature that many researchers had problems on using emulsions of linoleic acid and methyl linoleate as substrates to evaluate antioxidants, (75) and significant differences were found in the performance of antioxidants when tested in linoleic acid emulsions than with triacylglycerol emulsions.(63) A prolonged storage period was envisaged due to the fact that this study was conducted on a part-time basis and that such may be carried over 3 to 6 months or longer.

In the following sections the experiments carried out to evaluate the storage stability of a lipid substrate (sunflower oil) are reported. The composition of the gas in contact with the substrate and the storage temperatures were varied. Free fatty acid, peroxide value and iodine value determinations were carried out on the oil at regular intervals over an 18 week period to monitor the changes taking place during storage.

4.2 MATERIALS AND METHODS.

4.2.1 Materials

4.2.1.1 Sunflower oil treated as per method 3.2.2.3.

To remove natural antioxidants

4.2.1.2 High purity nitrogen gas.

Ex Air Products product number K296C. 11 kg per cylinder.

4.2.1.3 Edwards vacuum pump model Speedivac.

4.2.1.4 Three way valve system with attached syringe.

The valve system was used to draw vacuum from sample vials and introduce nitrogen into vials for the various experimental conditions. (APPENDIX 2)

4.2.1.5 Reaction vials.

Approximately 8ml volume with butyl septa and aluminium seals for sealing vials.

4.2.2 Methods

4.2.2.1 Preparation of substrate samples for storage test.

Fifty four reaction vials were stored at a constant temperature (25°C) and relative humidity (50%) for 24 hours to equilibrate. 18 reaction vials, labelled air samples, were filled with oil substrate and sealed with butyl septa and aluminium seal. A further 18 vials, labelled vacuum, were filled with oil substrate and sealed with butyl septa, the 3-way valve was attached to the seal via the syringe and a vacuum was applied to the headspace for 2 minutes with the aid of a vacuum pump. In the case of the vials to be stored under nitrogen, the oil substrate was first sparged with nitrogen, using a universal sintered tipped tube for ten minutes to displace any dissolved air. Vials were filled with the nitrogen sparged substrate without agitation, and sealed. A vacuum was applied to the head-space for two minutes followed by injection of nitrogen for two minutes through the three way valve system. These vials were labelled nitrogen. Headspace samples from each series were analyzed by gas chromatography. An initial analysis for free fatty acids, iodine value and peroxide value was conducted on the substrate prior to the storage tests.

4.2.2.2 Storage test of substrate samples.

Six samples each of vacuum, nitrogen and air were stored at 4°C, 23°C and 50°C. A sample of each condition from each storage temperature was removed after 14, 35, 70 and 126 days storage for analysis.

4.2.2.3 Free Fatty Acid determination (FFA) (76).

An accurately weighed 2g oil substrate was dissolved in 50ml of solvent mixture (1:1 ethanol : diethyl ether). The solution was titrated with 0.0100 normal sodium hydroxide using a micro burette and phenolphthalein indicator to a pink end-point which persisted for 10 to 15 seconds. The free fatty acid content was calculated from the following equation and expressed as % Oleic Acid. All test were carried in duplicate.

Calculation.

$$\% \text{ FFA (as Oleic Acid)} = \frac{\text{Titre(ml)} \times N \times 28.2^*}{\text{mass of sample}}$$

N = Normality of sodium hydroxide.

$$* \text{Factor } 28.2 = \frac{\text{Molecular mass oleic acid} \times 100}{1000}$$

4.2.2.4 Peroxide Value determination (77).

A 1g sample was weighed accurately into a 250ml Erlenmeyer flask and dissolved in 25ml of glacial acetic acid/chloroform solvent (2:1). 1ml of potassium iodide solution was added to the flask and the mixture was placed in the dark for exactly 1 minute. 35ml of distilled water was added to the flask and the reaction mixture titrated with 0.0020N sodium thiosulphate and fresh starch solution as indicator. A reagent blank was also

determined. The peroxide value was calculated from the following equation. All tests were carried out in duplicate.

Calculation.

$$\text{P.V.} = \frac{1000 \times (\text{titre sample} - \text{titre blank}) \times N}{\text{mass of sample.}}$$

titre = Volume of sodium thiosulphate used
during titration

N = Normality of sodium thiosulphate

The Peroxide Value is expressed in milliequivalents of peroxide oxygen per kilogram of oil sample.

4.2.2.5 Iodine Value (69).

Method as per 3.2.2.8 in Chapter 3

4.3 RESULTS AND CALCULATIONS.

4.3.1 Headspace analysis of vacuum, nitrogen and air substrate storage samples.

A 20ul headspace sample was drawn with an airtight gas micro syringe from each vial and injected into the gas chromatograph described in section 3.2.2.1

4.3.1.1 Vacuum substrate storage sample

No headspace sample could be drawn with the syringe for the vacuum sample since the vial was under vacuum and the syringe plunger kept springing into its barrel each time a headspace sample was to be drawn.

4.3.1.2 Nitrogen substrate storage sample

Gas chromatographic analysis of the headspace of the nitrogen substrate storage sample gave a nitrogen to oxygen ratio of

127.3. A typical chromatogram of the headspace of the nitrogen substrate is shown in Figure 4.1 chromatogram a.

4.3.1.3 Air substrate storage sample

Headspace analysis of the air substrate sample gave a nitrogen to oxygen ratio of 3.94 and a chromatogram of the headspace is shown in Figure 4.1 chromatogram b.

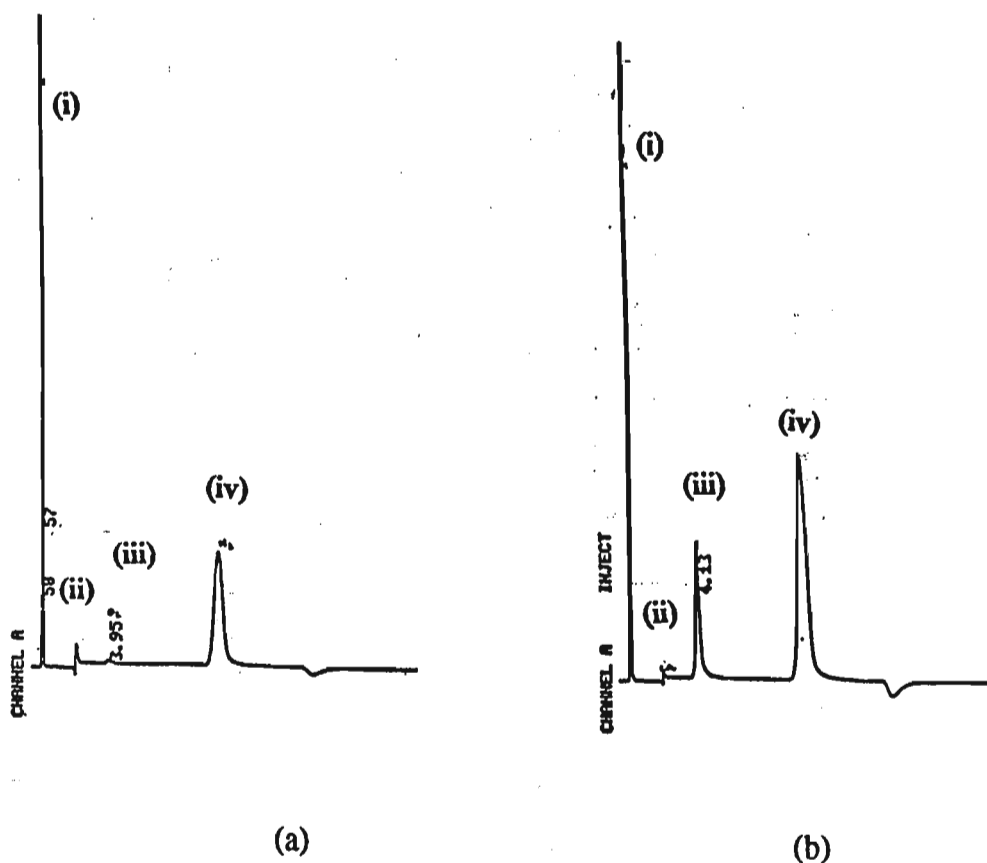


Figure 4.1 Chromatograms of headspace analysis of substrate storage samples. Peaks: (i) = composite air (ii) = carbon dioxide (iii) = oxygen (iv) = nitrogen.

4.3.2 Analysis of substrate storage samples.

Substrate storage samples packed under vacuum, nitrogen and air, stored at 4°C 23°C and 50°C were analyzed for free fatty acids, peroxide value, and iodine value.

4.3.2.1 Free Fatty Acid content of stored samples

Free Fatty Acids were determined for samples stored under the different conditions and at various temperatures after 14, 35, 70 and 126 days. Table 4.1 illustrates the free fatty acid of the substrate after storage and Figures 4.2, 4.3 and 4.4 show a graphical representation of the free fatty acids from the various conditions over the storage period for 4°C, 23°C and 50°C respectively.

Table 4.1 Free Fatty acids of substrate storage samples

Storage period	Conditions	4°C	23°C	50°C
days		%	%	%
0	-	0.06	0.06	0.06
14	nitrogen	0.07	0.08	0.11
	vacuum	0.07	0.08	0.10
	air	0.08	0.09	0.12
35	nitrogen	0.08	0.09	0.11
	vacuum	0.08	0.09	0.13
	air	0.09	0.10	0.18
70	nitrogen	0.10	0.11	0.15
	vacuum	0.10	0.12	0.19
	air	0.11	0.16	0.37
126	nitrogen	0.11	0.13	0.14
	vacuum	0.13	0.20	0.21
	air	0.21	0.26	0.41

These results were plotted as graphs of F.F.A versus time (Days) in Figures 4.2 - 4.4.

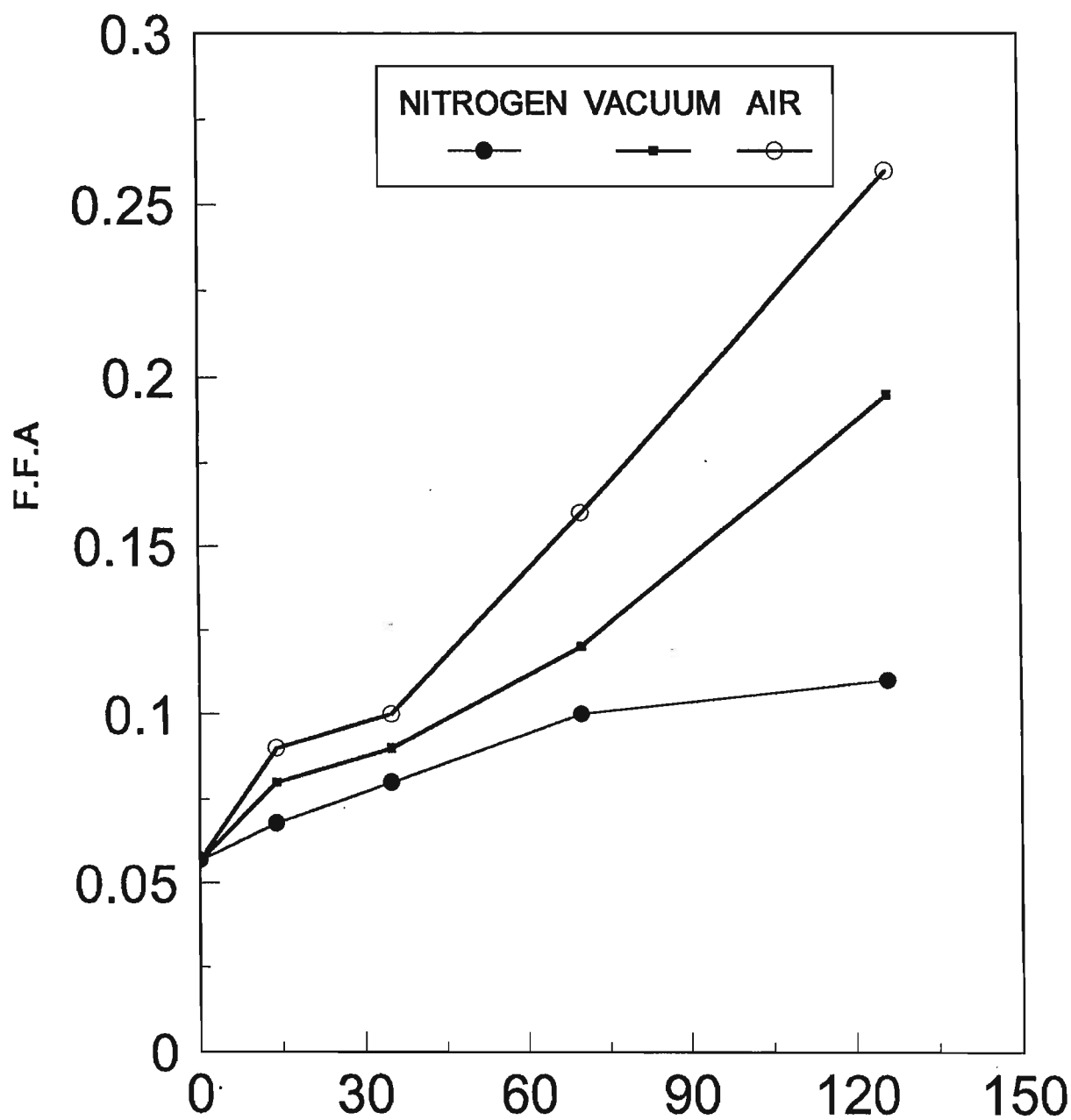


Figure 4.2 Free Fatty Acids of substrates stored at 4°C.

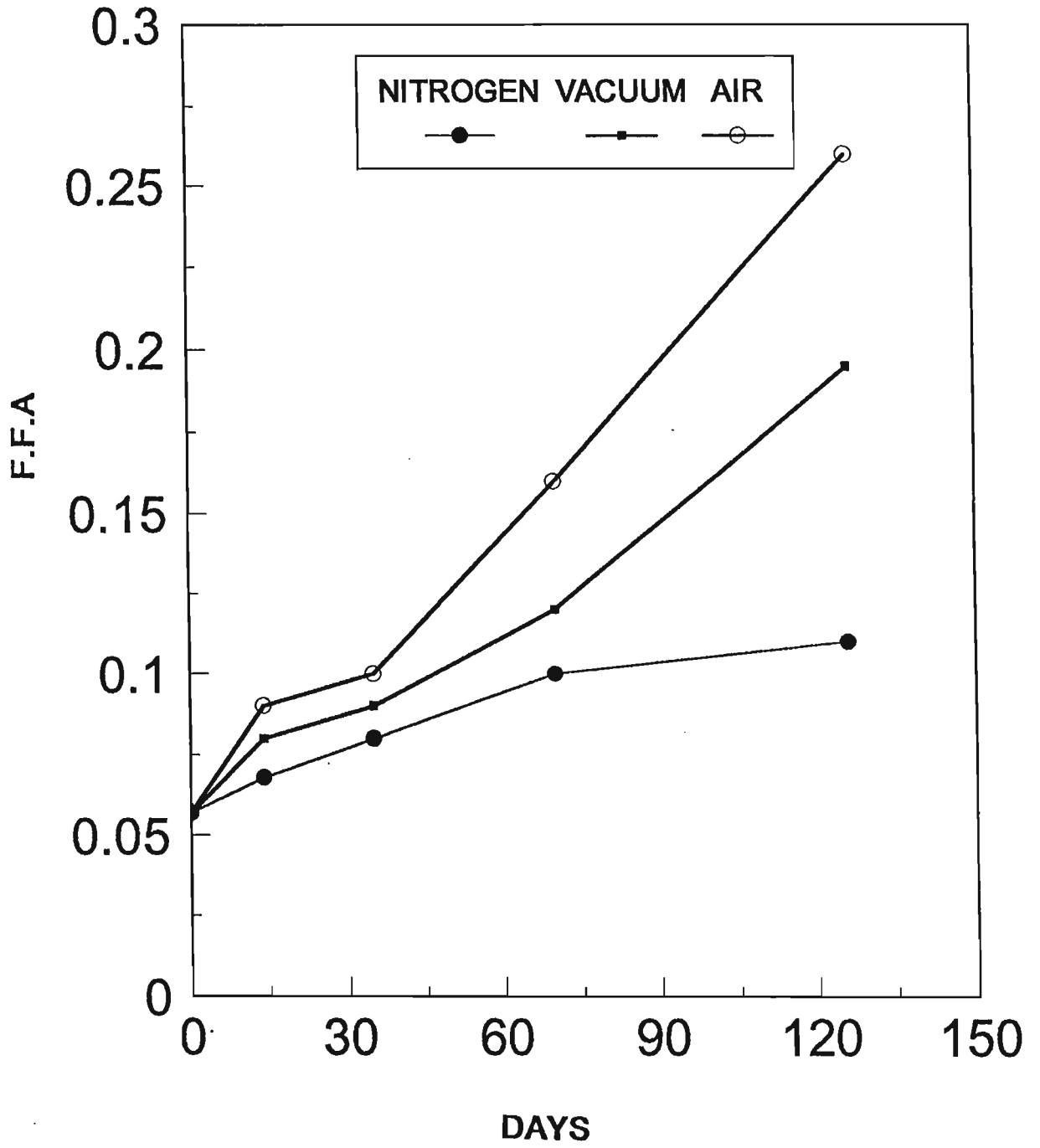


Figure 4.3 Free Fatty Acids of substrates stored at 23°C

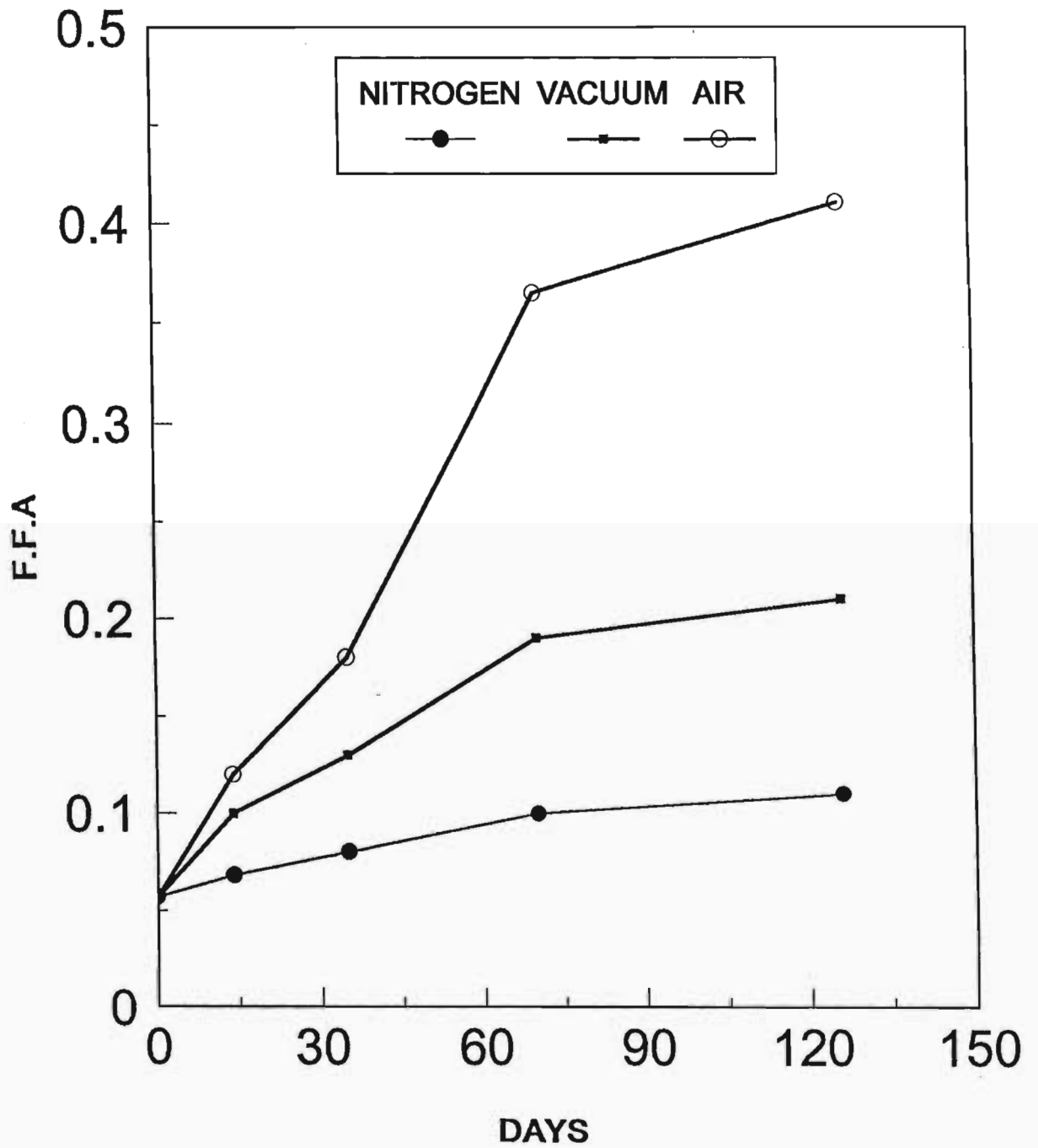


Figure 4.4 Free Fatty Acids of substrates stored at 50°C

4.3.2.2 Peroxide Value of substrate stored samples.

Peroxide Value was determined for samples stored under the different conditions and at various temperatures after 14, 35, 70, 126 days. Table 4.2 illustrates the peroxide value of the substrate after storage and Figures 4.5, 4.6 and 4.7 show the graphical trend of the peroxide values from the various conditions over the storage period for 4°C, 23°C and 50°C respectively.

Table 4.2 Peroxide Value of substrate storage samples

Storage period	Conditions	4°C	23°C	50°C
days		meq/kg	meq/kg	meq/kg
0	-	0.09	0.09	0.09
14	nitrogen vacuum air	1.3 1.4 2.1	1.1 1.2 1.2	2.1 1.3 2.7
35	nitrogen vacuum air	1.5 1.8 5.9	1.3 1.4 3.8	2.3 2.4 11.7
70	nitrogen vacuum air	2.2 3.1 13.8	1.8 2.6 7.9	2.7 4.3 29.5
126	nitrogen vacuum air	2.9 4.9 23.6	2.2 3.0 12.5	2.8 6.8 14.9

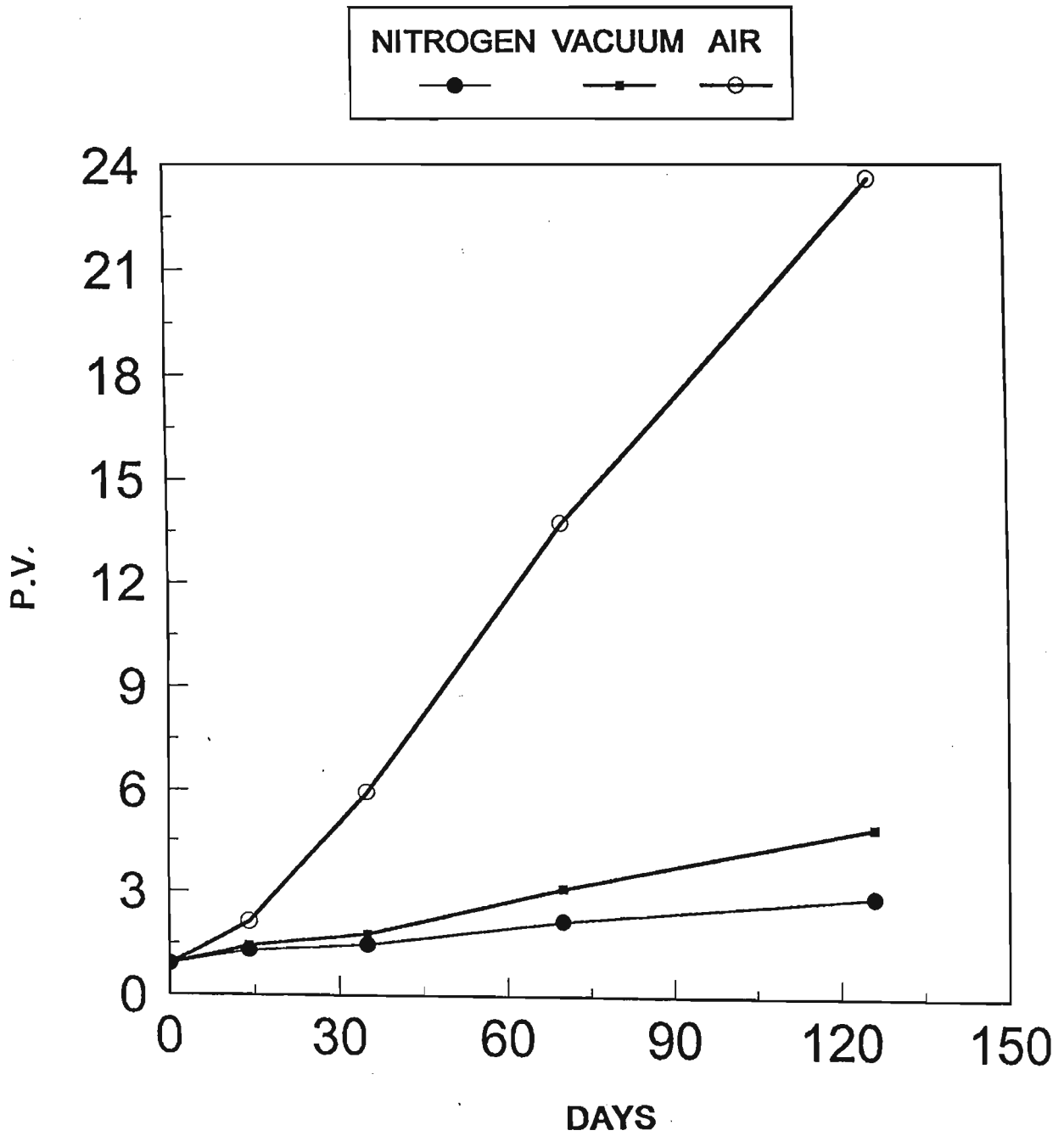


Figure 4.5 Peroxide Value of substrates stored at 4°C.

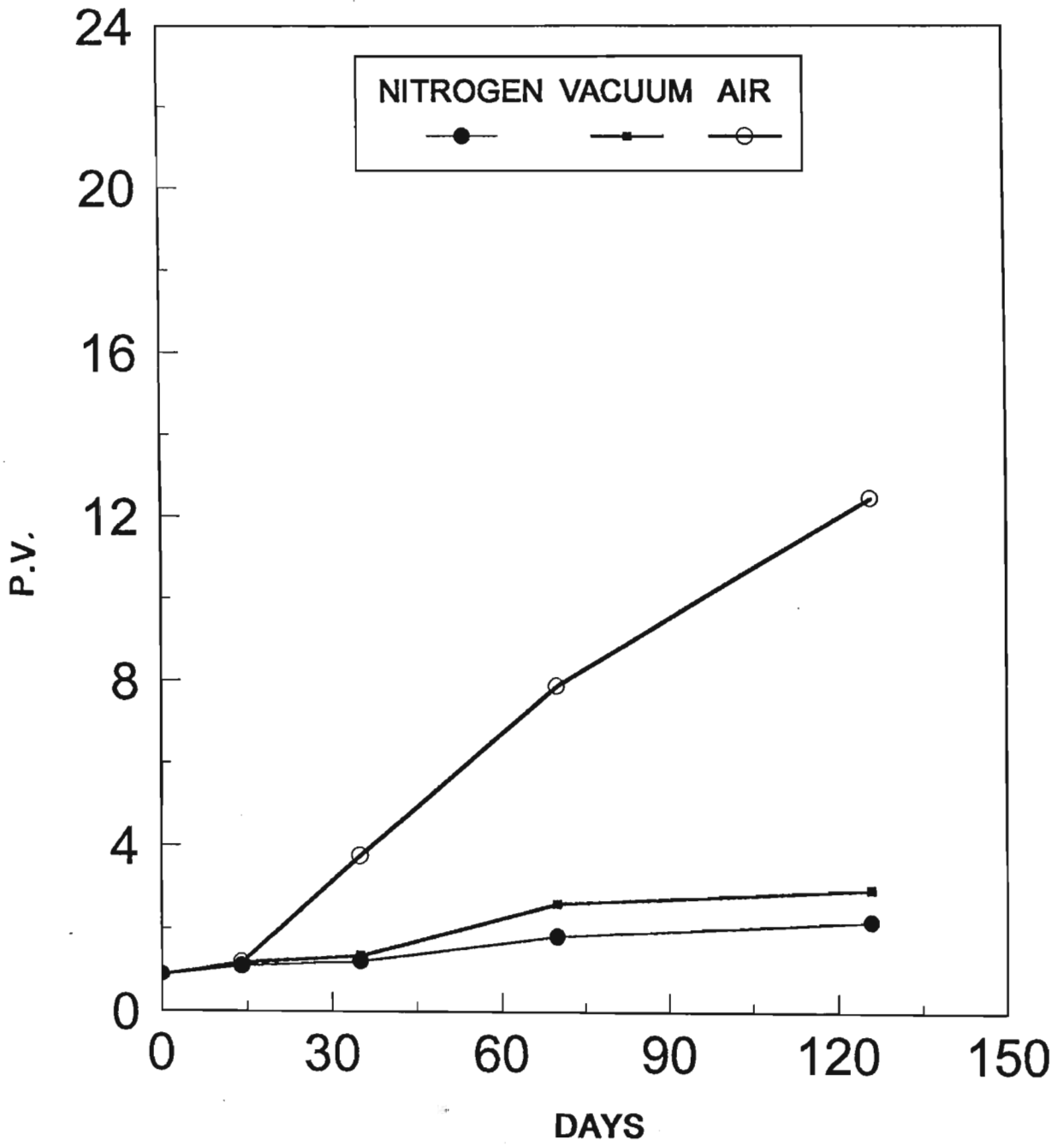


Figure 4.6 Peroxide Value of substrates stored at 23°C.

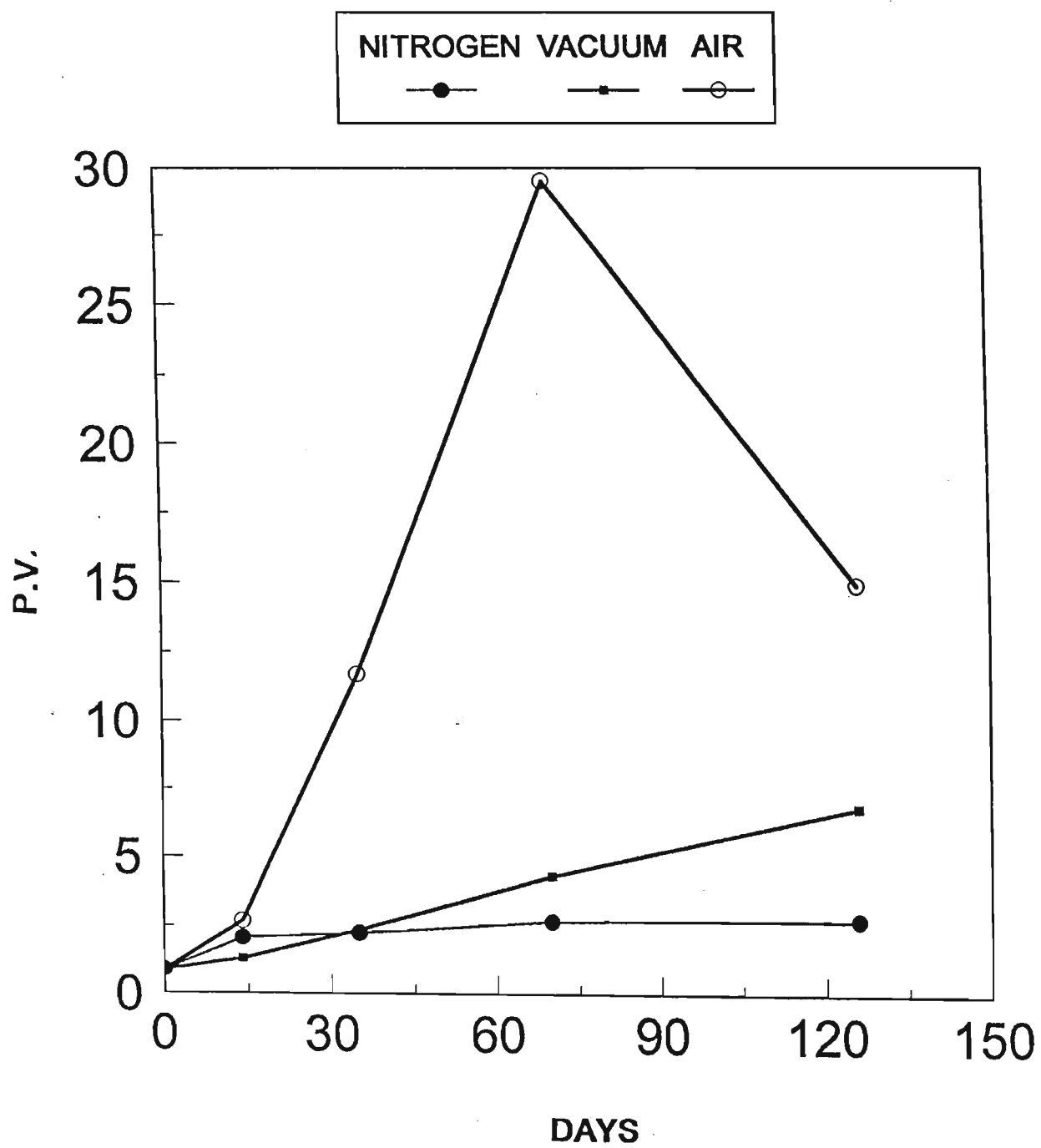


Figure 4.7 Peroxide Value of substrates stored at 50°C.

4.3.2.3 Iodine Value of stored substrate samples

Iodine Values were determined for samples stored under the different conditions and at various temperatures after 14, 35, 70 and 126 days. Table 4.3 illustrates the iodine value of the substrate after storage and Figures 4.8, 4.9 and 4.10 show a graphical trend of the iodine values of the substrate from the different conditions over the storage period for 4°C, 23°C and 50°C.

Table 4.3 Iodine Value of substrate storage samples.

Storage period	Conditions	4°C	23°C	50°C
days				
0	as is	136.2	136.2	136.2
14	nitrogen	136.7	134.9	134.1
	vacuum	134.1	134.4	134.6
	air	134.7	134.5	134.0
35	nitrogen	136.0	134.1	132.6
	vacuum	134.4	134.4	134.2
	air	134.9	134.6	123.9
70	nitrogen	134.1	130.6	126.7
	vacuum	131.3	131.2	125.7
	air	129.3	128.1	122.5
126	nitrogen	130.1	130.0	126.0
	vacuum	131.1	126.8	122.1
	air	127.2	120.4	109.0

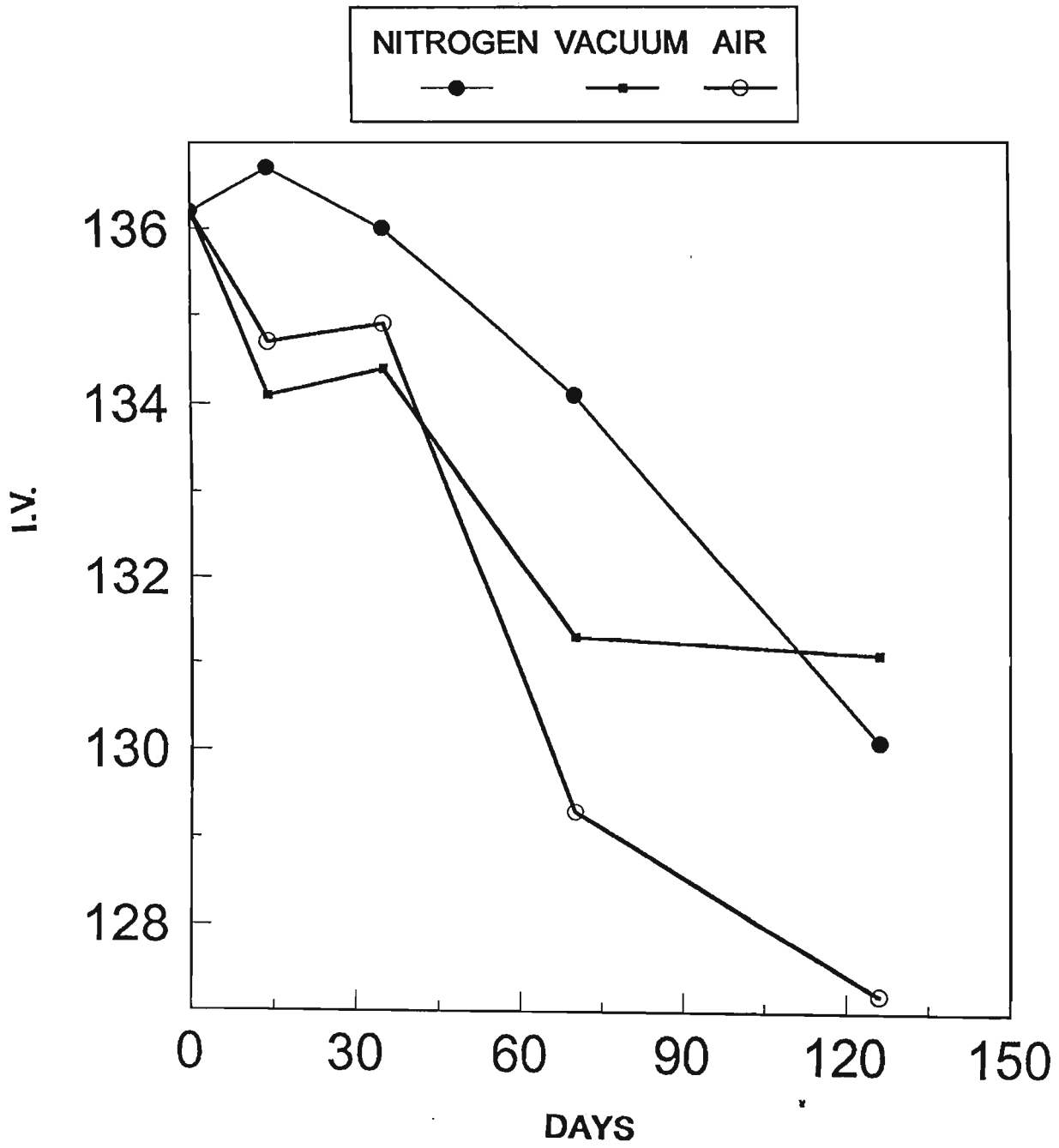


Figure 4.8 Iodine value of substrates stored at 4°C.

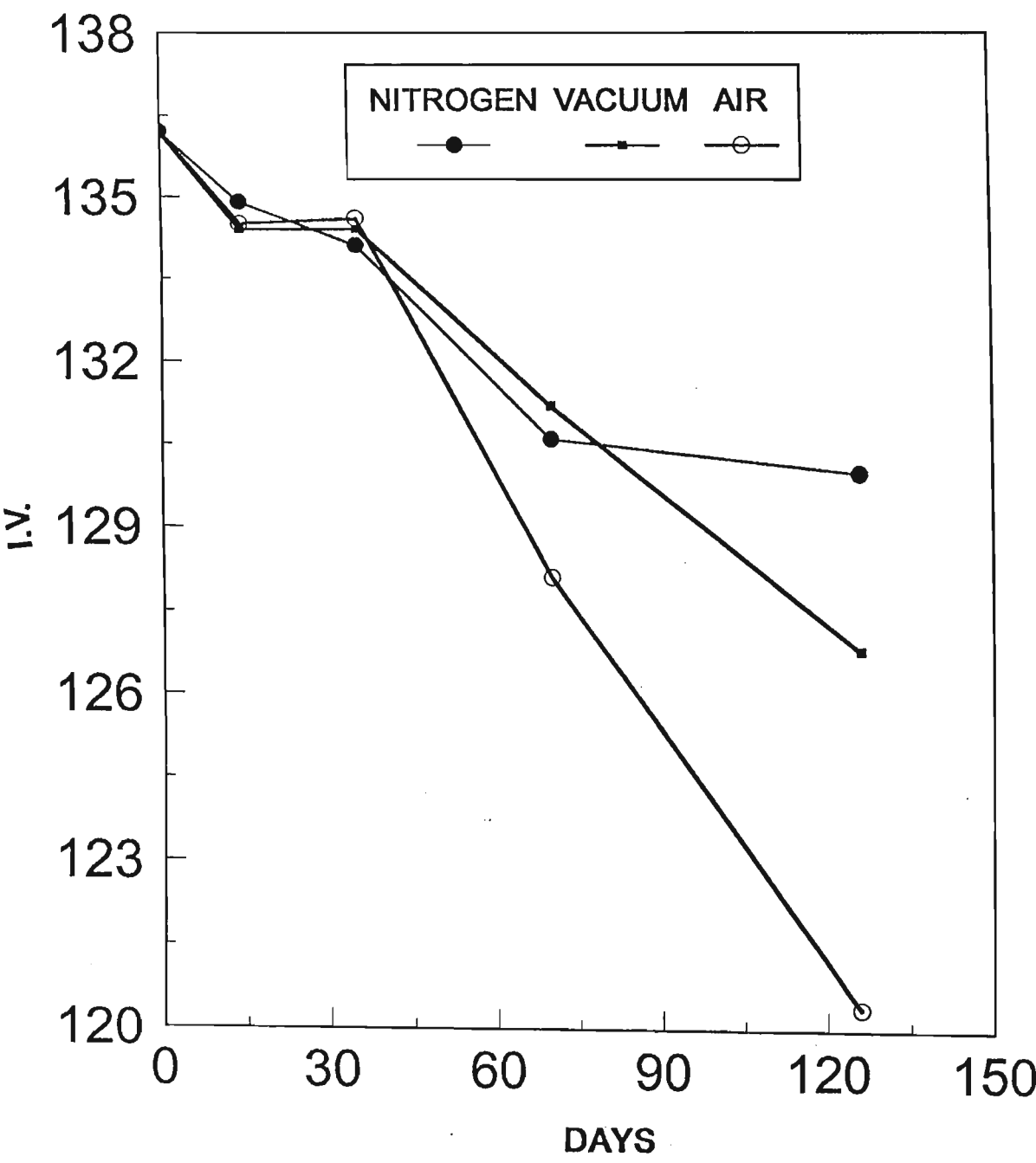


Figure 4.9 Iodine value of substrates stored at 23°C.

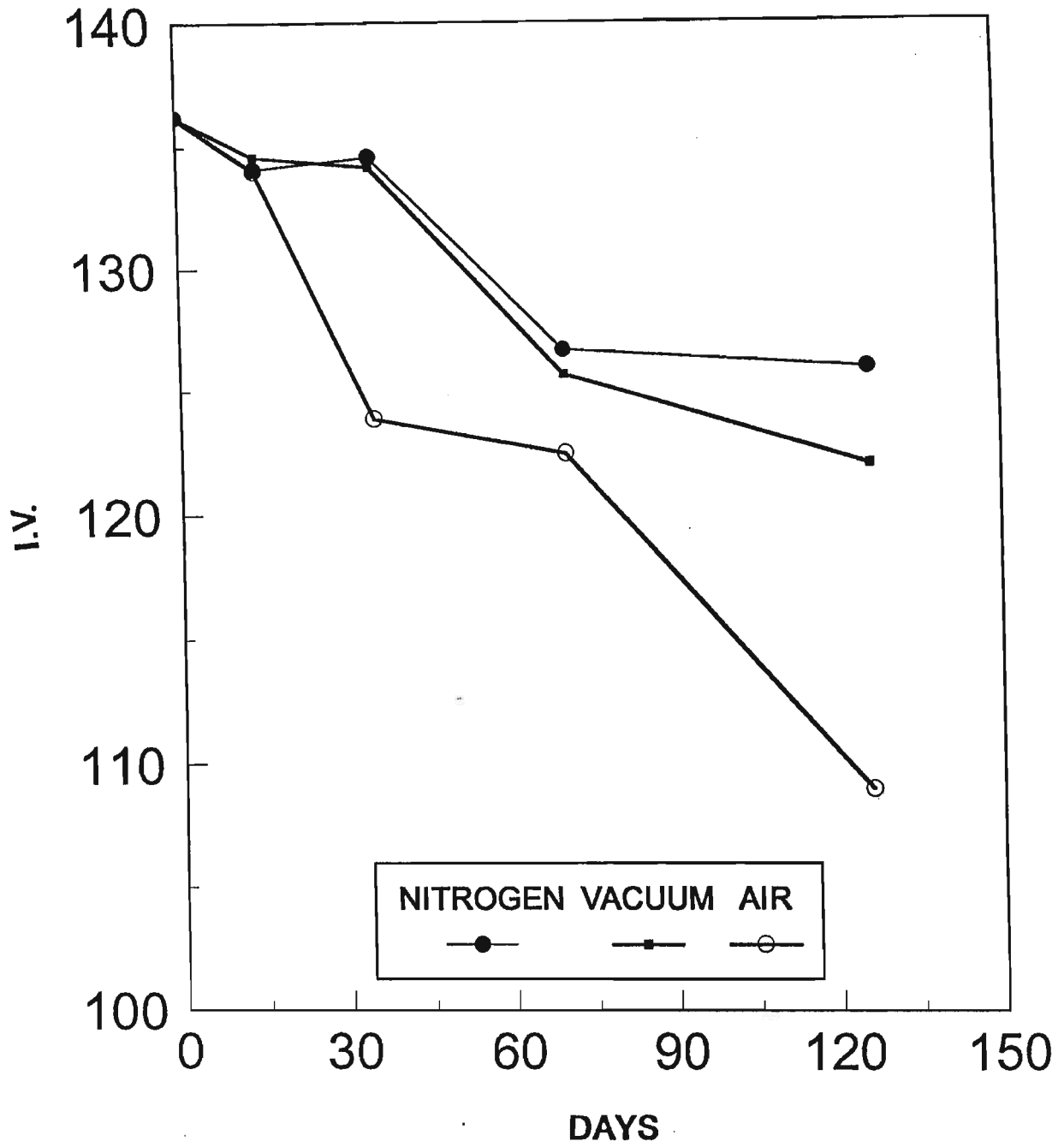


Figure 4.10 Iodine value of substrate stored at 50°C.

4.4 DISCUSSION

The gas chromatographic analysis for the respective headspace storage conditions showed that in the air headspace the N_2/O_2 was 3.89, which is similar to that of normal air. For the nitrogen headspace a very small oxygen peak was detected to give a N_2/O_2 of 127.3 while for the vacuum headspace no air sample could be drawn indicating that the sample was under vacuum.

Free fatty acid analysis under the various temperatures clearly show the substrate stored under nitrogen to be lower in FFA than those stored in vacuum and air. All the air substrate samples were found to show sharp increases in free fatty acids after 30 days storage.

Peroxide values were found to be very low in the nitrogen and vacuum substrate samples over the storage period which is probably due to the lack of residual air present for oxidation. Peroxide values for the air samples showed very sharp increases between 10 and 15 day storage at all temperatures with a drop in peroxide value observed with samples stored at 50°C, probably resulting from breakdown of oxidised products.

Iodine values were lower in all air samples, with lowest value recorded for the samples stored at 50°C, while iodine values of nitrogen and vacuum substrate samples were found to be acceptable at this high temperature. Very similar iodine values were recorded for nitrogen and vacuum substrate samples stored at 4°C and 23°C.

Both nitrogen and vacuum headspace conditions showed promise in the storage of substrate samples. However, it was difficult to establish the level of residual oxygen (due to dissolved air) in the vacuum samples. Also it was impossible to withdraw headspace samples using this storage condition. Therefore, it was decided to use the nitrogen storage condition for substrates to be used in further investigations.

CHAPTER 5

ANTIOXIDANT ACTIVITY OF MAILLARD REACTION PRODUCTS.

5.1 INTRODUCTION.

Various constituents in foods undergo a multitude of interactions during cooking. The products formed by such thermal reactions change the flavour, colour, and taste of cooked foods. The primary pathways responsible for these phenomena is the formation of Maillard reaction products, which involves the reaction between amines and carbonyl compounds. This reaction is also frequently exploited and applied in the food industry to modify the colour and flavour of commercial products (78).

The Maillard reaction is one of the most challenging areas of food chemistry. In the initial stages, the reaction represents a simple nucleophilic addition between the NH_2 group of amino compounds, such as amino acids and proteins, and electrophilic carbonyl groups of reducing sugars. In food technology there is a need to better understand the reaction pathways that result in the desirable and undesirable attributes in finished products, such as the flavour profiles of baked and over roasted products (79).

It is essentially the monosaccharides glucose and fructose that form γ -pyranones; the disaccharides such as maltose or lactose yield β -pyranones as major products which subsequently transform to stable isomaltal glycosides (79). The problem of identifying synthesis pathways in the Maillard reaction is complicated by the fact that several reaction pathways and even reverse reaction pathways are operative simultaneously, and some products may be formed from more than one type of intermediate (79). Whenever foods containing protein and reducing sugars are heated, even at relatively low temperatures and for short periods of time, Maillard reactions products are formed (80). The effect of sugar structure on extent of browning has been found to decrease in the order of D-xylose > L - arabinose > hexoses (D -

galactose; D - mannose, D - glucose and D - fructose) > disaccharides (maltose, lactose and sucrose). D - fructose is much less reactive than the aldoses because of the different mechanism followed by ketose sugars. The degree of pigment formation from a particular sugar is directly proportional to the amount of open chain (free carbonyl) sugar present in the product which strongly suggest that the amine reacts with the open chain form (80). In a glucose-glycine model system containing 65% water and stored at 65°C, a rapid increase in colour formation was evident as the glucose-glycine ratio decreased from 10:1 or 2:1 to 1:1 or 1:5 (81). When Maillard browning is undesirable in food systems it can be inhibited by removing one of the reactants, usually the reducing sugar for protein-rich foods or the amino compounds for carbohydrate-rich foods (39).

The Maillard reaction takes place not only during the processing but also on storage of protein foods containing reducing carbohydrates or carbonyl compounds. Since many of the chemical reactions involved in the Maillard reaction possess a high activation energy, they are markedly enhanced during cooking, heat processing, evaporation, drying and extrusion cooking (82). The latter is a high temperature continuous food reactor which causes food ingredients to interact under temperature, pressure and shear via numerous mechanisms and induces the formation of Maillard reaction products (83).

Extruders in general consist of a fixed metal barrel through which material is transported. The barrel contains one or two screws that convey the food material from the feed end of the barrel to the die exit, which determines final shape of product. Heat can be applied to the barrel, but heat generated by friction and shear forces may also be sufficient to cook the material. The dimensions and geometry of the barrel, the screw compression ratio and position of elements on screw are variables which affect shear and pressure within the extruder. The speed of the screw rotation and the length of residence time within the extruder also affects the degree of shear (84). A general diagram of an extruder is given in Figure 5.1

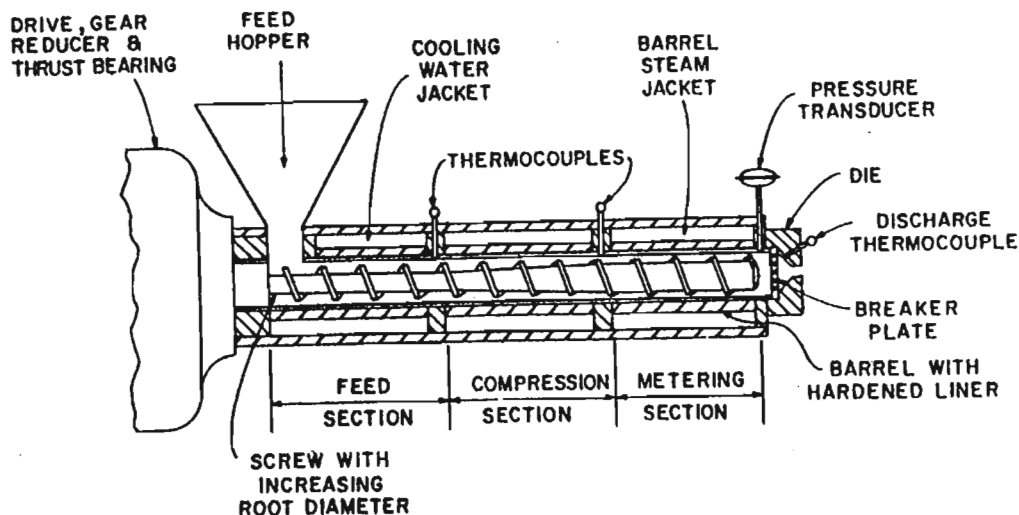


Figure 5.1 Cross-section of a typical food extruder (85).

During extrusion of a food material the environment inside the extruder is ideal for Maillard reaction products to form. The chemistry involved in Maillard reactions is complex but the final products are brown insoluble pigments. The melt within the extruder often contains many free amino groups and also sources of potential carbonyl groups to form brown extrudates, whose degree of browning varies quite markedly with both the processing conditions and the nature of the ingredients (86).

Studies of the changes that proteins undergo during extrusion processing have mainly concentrated on the free amino groups of lysine. It has been established that the concentration of these groups decreases subsequent to extrusion. Available lysine decreases by up to 50% depending on the severity of the processing and on the reactivity of the other ingredients. The colour of the product can also markedly be affected (87).

The Maillard reaction resulting in loss of biologically available lysine decreases the nutritive value of proteins. This loss of lysine also affects the molecular structure of the proteins, since both intermolecular and intramolecular covalent bonds between protein polypeptide chains can occur.

This may hinder hydrolysis by proteolytic enzymes and effectively diminish the digestibility of the protein as a whole (88).

In this investigation extrusion technology was utilised to develop reaction products in soya flour under very mild extrusion conditions (ie low shear and pressure and at moderate temperatures) with the inclusion in the extrusion mix of reducing sugars to promote the Maillard reaction. Analysis of the respective concentration of reducing sugars prior to and after extrusion as well as available lysine content can give an indication of the reactivity of the components responsible for the formation of Maillard reaction products. The development of antioxidant activity as detected by the measurement of induction periods by the nitrogen oxygen ratio method of Ross and de Muelenaere and Rancimat method will also be determined

5.2 **MATERIALS AND METHODS**

5.2.1 **Materials**

5.2.1.1 **Sunflower oil stripped of natural antioxidant as per chapter 3.**

Section 3.2.2.3 was used as substrate.

5.2.1.2 **Fullfat soyabean flour:**

Whole soyabeans were ground in an Alpine mill fitted with a 750um screen.

5.2.1.3 **Fullfat soyabean flour with glucose.**

1 kg of glucose B.P. was mixed with 10 kg of soyabean flour in a Hobart mixer.

5.2.1.4 **Fullfat soyabean flour with fructose.**

1 kg of fructose B.P. was mixed with 10 kg of soyabean flour in a Hobart mixer.

5.2.2 Methods

5.2.2.1 Determination of moisture content (89).

Overnight oven method.

Samples of about 5g were accurately massed into pre-weighed aluminium dishes. The dishes were placed in an air oven which was kept at a constant temperature of 105°C. After 16 hours the dishes were removed, allowed to cool in a desiccator and reweighed until constant mass. The moisture content was calculated from the difference in mass.

$$\% \text{ Moisture} = \frac{\text{Difference in mass} \times 100}{\text{mass of sample}}$$

5.2.2.2 Extraction of lipids (90).

Equipment and reagents

- i) Soxhlet apparatus complete with condenser and heating mantle
- ii) 250ml boiling flasks with side neck Q/F B14
- iii) Defatted cotton wool
- iv) Whatman extraction thimbles
- v) HPLC grade n-Hexane
- vi) Whatman no. 1 filter paper
- vii) Nitrogen gas ex Air Products

Procedure

The soya beans were ground in a Alpine mill fitted with a 750um screen. 5g of sample was massed accurately on to Whatman no.1 filter paper which was folded and placed into an extraction thimble. The thimble was plugged with defatted cotton wool and placed in the Soxhlet extractor.

150ml of n-Hexane was added to the premassed round bottom boiling flask. The flask was connected to the Soxhlet extractor and condenser and heated over a heating mantle for 16 hours. The extraction was carried out under a stream of nitrogen by placing a pasteur pipette with a very fine tip through the side neck of the flask. Any loss of solvent was replaced during extraction.

The hexane extract in the flask was evaporated on a hot waterbath in a fume cupboard. The flask was dried to a constant mass in a air oven at 105°C. The percentage lipids in the sample was calculated from the mass of the extracted lipids.

$$\% \text{ lipids} = \frac{\text{Mass of extracted lipids} \times 100}{\text{Mass of sample}}$$

5.2.2.3 Determination of available lysine. (Carpenter method) (91)

Reagents.

- i) Fluorodinitrobenzene (FDNB) reagent
2-4 Dinitrofluorobenzene Reidel de Haen prod.
no. 33253
FDNB 0.3ml
Absolute ethanol 12.0ml
- ii) 8.1 M hydrochloric acid
HCl 80.0ml
Distilled water 20.0ml
- iii) 8% (w/v) sodium bicarbonate solution.
- iv) Diethylether
- v) 1 M hydrochloric acid
- vi) Phenolphthalein indicator

- vii) 1 M sodium hydroxide
- viii) pH 8.5 buffer
 - 8% (w/v) sodium bicarbonate 19 parts
 - 8% (w/v) sodium carbonate 1 part
- ix) Methyl chloroformate
- x) Conc. hydrochloric acid
- xi) DNP - lysine stock solution. (Dinitrophenol-lysine).
 - DNP - lysine 100ug
 - 1 M HCl 1ml

This solution contains equivalent of 45.5ug lysine/ml.
- xii) DNP - lysine standard solutions.
 - 0.5; 1.0; 2.0; 3.0; and 4.0ml of DNP-lysine stock solution. were made up to 10ml with 1 M hydrochloric acid.

Procedure.

8mls of 8% sodium bicarbonate was added to an amber round bottom flask. 1g of finely ground sample was added to the flask with 0.3ml FDNB dissolved in 12ml absolute ethanol. The flask was shaken gently for 2 hours. Care was taken not to work in direct sunlight. The FDNB was stored in the dark when not in use.

The ethanol was evaporated off in a boiling waterbath until no more evidence of effervescence was observed. 24ml of 8.1 M hydrochloric acid was gently added to the flask. The flask with condenser was gently refluxed on a heating mantle for 16 hours.

The flask was removed and cooled with ice water for 1 hour. The contents was filtered with Whatman 00.40 filter paper into a 200ml amber volumetric flask and made up to mark with distilled water.

A 20ml aliquot of filtered solution was diluted to 100ml in an amber 100ml volumetric flask. 4ml of this diluted filtrate was added to a 20ml stoppered test tube. The contents was extracted twice with 10ml diethyl ether. The ether was removed by vacuum suction and immersion of test-tube in hot water.

10ml of 1 M hydrochloric acid was added to the tube and the optical density read against 1 M HCl blank in a Philips DB 20 spectrophotometer at 425nm wavelength.

A further two 4ml aliquots of diluted filtrate were pipetted into two glass test tubes and extracted with diethyl ether as described above. The contents of one of the tubes was then poured into a small Erlenmeyer flask. 10ml of 1 M hydrochloric acid was added to this flask together with 3 drops of phenolphthalein indicator, and the solution then titrated with 1 M sodium hydroxide. The sodium hydroxide volume needed to neutralise the acidic solution was noted and the contents of flask discarded.

The same volume of sodium hydroxide used in the titration above was added to the second glass stoppered tube, together with 2ml buffer solution. (pH 8.5) 0.05ml methyl chloroformate was then carefully

added to the tube. This was done in the fume cupboard. The tube was stoppered, shaken and left to stand for 10 minutes.

0.75ml conc. hydrochloric acid was added slowly to the tube, the contents of the tube again extracted with 2x10ml aliquots of diethyl ether. The ether was removed under vacuum and by immersion in hot water. The solution was made up to 10ml with distilled water. This solution was regarded as the blank and read on the spectrophotometer at 435nm using distilled water as reference.

Standard solutions were made of DNP-lysine stock solution and read at 435nm. A standard curve was plotted from which the available lysine content of the experimental samples was determined. The concentration of available lysine in the original sample was expressed as available lysine per 100g crude protein. The available lysine content was calculated using the following formula.

$$\text{Available lysine g/100g protein} = \frac{\text{ug lysine} \times 99}{80 \times \text{sample wt.} \times \% \text{ protein}}$$

Determination of Protein content (92)

5.2.2.4 Determination of reducing sugars (93).

Apparatus

Hot plate

250ml beaker

600ml beaker

2 x 250ml Erlenmeyer flasks

Balance

2 x 15ml bulb pipettes

2 x 1000ml volumetric flasks

Filter paper Whatman no. 4

Funnels

Reagents

For the preparation of Fehlings Solution A:

Copper Sulphate Pentahydrate ($\text{CuSO}_4 \cdot 5 \text{H}_2\text{O}$)

For the preparation of Fehlings Solution B:

Potassium Sodium tartrate tetrahydrate

($\text{C}_4\text{H}_4\text{KNaO}_6 \cdot 4\text{H}_2\text{O}$)

Sodium Hydroxide (NaOH)

Dextrose - A.R.

Deionised Water.

0,1% Methylene Blue indicator.

PREPARATION AND STANDARDIZATION OF FEHLINGS A & B (93)

i) Preparation of Fehlings Solution A:

Dissolve 69,30g of $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ and dilute to mark in a 1000ml volumetric flask with distilled water.

ii) Preparation of Fehlings Solution B:

Note: Due to the exothermic reaction during the preparation, cooling is essential.

Dissolve 346,00g of Potassium Sodium Tartrate and 100,00g NaOH in a 600ml beaker cooling in a basin of water. Transfer solution into a 1000ml volumetric flask and bring up to mark once the solution reaches room temperature with distilled water.

Filter and transfer to a tinted bottle.

- iii) Preparation of 0,1% Methylene Blue indicator.
Dissolve 0,50g of Methylene Blue indicator in 100ml of alcohol.
Add 400ml of de-ionised water and mix thoroughly.
- iv) Standardisation of Fehlings Solution A & B
Note: Every batch of Fehlings solution made must be standardised prior to use.
Weigh out accurately 1,2500g dextrose A.R. (that has been previously dehydrated at 105°C for 2-3 hours) to 4 decimal places.
Dissolve in a 250ml volumetric flask and make up to mark with de-ionised water.

Procedure.

Dispense 15ml of each Fehlings solution A and B using a bulb pipette into a 250ml Erlenmeyer flask. Add 2 bumping beads and 3-5 drops of 0,1% Methylene blue indicator.

Start heating and immediately add the dextrose solution from a burette to about 0,5ml before the end point.

Bring the mixture to a boil and boil gently for 2 minutes.

As boiling continues complete titration within one (1) minute by adding the dextrose solution dropwise until the blue colour disappears.

Record the volume of the dextrose solution used and calculate the Fehlings Factor as follows:

$$\text{Fehlings Factor} = \frac{\text{Mass of dextrose x vol. of dextrose solution used}}{\text{Total vol of dextrose solution x vol of Fehlings A\&B}}$$

FEHLINGS METHOD FOR ESTIMATION OF REDUCING SUGARS (93).

Procedure.

Weigh 6,00g sample into a 250ml beaker --> record the mass. Bring up to 150g with de-ionised water and stir to dissolve (4% solution). Dispense 15ml each of standardised Fehlings solutions A and B into a 250ml Erlenmeyer flask using a bulb pipette.

Add 2 bumping beads and 3-5 drops of a 0,1% Methylene blue indicator solution. Start heating and immediately add the sample solution from a burette to about 0,5ml before the end point.

Bring the mixture to a boil and boil gently for 2 minutes. As boiling continues complete the titration within 1 minute by adding the sample solution dropwise until the colour disappears. The end point is clear solution with red precipitate. Record the volume of solution used.

Calculation:

% reducing sugars =

$$\frac{\text{vol of mixed Fehlings A\&B x Fehlings Factor x vol of sample x 100}}{\text{Titer x Mass of sample.}}$$

5.2.2.5 Determination of glucose and fructose by HPLC (94).

HPLC Parameters.

Column : Phenomenex

Type : Rexex monosaccharide 300 x 78mm

Mobile phase : 100% water HPLC grade
Flow rate : 0,6ml / min
Temperature : 85°C
Detector : Beckman 156 R I Detector
Pump : Beckman 112 solvent delivery system
Integrator : H.P. 3390A

Procedure.

Samples were prepared by allowing 5g (accurately massed) to mix in 80ml hot water in a 100ml volumetric flask. The mixture was cooled and made up to mark. A 10ml aliquot was filtered through a 0,22µ Millex - GS sterilising filter unit (waters). The aqueous solution was injected together with glucose and fructose standard solutions to identify the peaks and to calculate the concentration of the individual sugars from the integration counts.

5.2.2.6 Extrusion Processing.

Soya flour with and without sugars were processed in a Cleextral BC 45 twin-screw extruder. The extruder consisted of a smooth barrel surrounded by three 7kW induction heaters. The temperature of the exiting product was measured by means of a thermocouple probe (J-type) inside the die plate. Four other thermocouples measured the barrel temperature. The location of the thermocouples on the extruder is indicated in Figure 5.2.

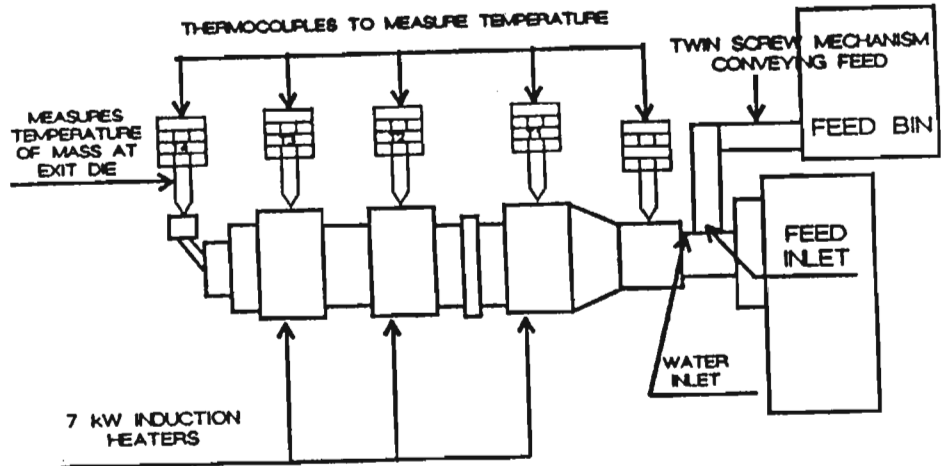


Figure 5.2 Diagram of Extruder to indicate position of thermocouples

The total length of the co-rating screws was 1000mm and the screw configuration from the inlet to the die of the extruder as indicated in Tables 5.3 and 5.5 remained unchanged throughout the extrusions.

The air gap between the die and the end of the screws was 10mm. The single aperture of 1.8mm diameter in the die plate was use during extrusion. Material was fed continuously into extruder from the feed hopper by a twin-screw system at a rate of 400g per minute.

5.2.2.7 Determination of induction periods of lipids extracted from unextruded and extruded soya flour.

Procedure

The lipids were extracted from unextruded and extruded soya flours with n-hexane under a stream of nitrogen. The hexane was evaporated in a rotary evaporator at 45°C. After removing solvent the lipids were stored in reaction vials under nitrogen at -16°C until required. The induction period of the respective lipids were determined by the Ross de Muelenaere method and the Rancimat method by constructing tangents to the horizontal and vertical portions of the curves. The induction period is taken from the start of the oxidation to the point which is perpendicular to the intersect of the tangents.

5.3 RESULTS AND CALCULATIONS.

5.3.1 Solvent extraction of lipids from fullfat soyabean flour.

Several solvents were used to extract the lipids from soya flour to establish the most suitable solvent for maximum extractable lipids. The boiling points of the solvents ranged from 30°C to 70°C. The lipids were extracted with H.P.L.C. grade solvents in a Soxhlet apparatus under a stream of nitrogen. The solvent was removed from the extracted lipids under nitrogen and reduced pressure at 50 - 55°C using a rotary evaporator instead of the waterbath as described in section 5.2.2.2.

The mass of the extracted lipids was expressed as a percentage of the mass of the soya flour used. From the results in Table 5.1 n-Hexane was chosen as the suitable solvent for extraction of lipids from unextruded and extruded soya flour.

Table 5.1 Extraction of lipids from soya flour by various solvents.

Solvents	*Total extractable lipids
Petroleum ether 30-60°C	17.8
Diethyl ether	17.1
Methanol	9.7
n-hexane	18.4

*Average value of triplicate determinations.

5.3.2 Extrusion experiments

5.3.2.1 Extrusion of fullfat soyabean flour

Extrusion conditions were initially established with standard soyabean flour using a screw configuration and low pressures to reduce shear effects to a minimum, while moderate temperatures were applied to induce formation of Maillard reaction products. Oxidation of hexane extracted lipids from extruded and unextruded soyabean flours showed very little differences. Table 5.2 shows the Nitrogen to Oxygen ratios obtained from the oxidation of the lipids from extruded and unextruded soyabeans. Figure 5.3 shows the oxidation of the lipids and their induction periods. Table 5.3 shows the extrusion parameters used.

Table 5.2 Nitrogen to Oxygen ratio calculated for lipids of extruded and unextruded soya flour

Hours	Unextruded soya N_2/O_2	extruded soya N_2/O_2
0	3.88	3.86
16	3.92	3.90
24	3.95	3.93
40	4.06	3.98
44	4.31	4.29
46	4.53	4.77
48	5.12	5.46
64	17.68	18.93

A plot of N_2/O_2 against hours was drawn as per Figure 5.3a for unextruded soya flour and Figure 5.3b for extruded soya flour and the induction period of the lipids obtained from the tangents at the inflection point.

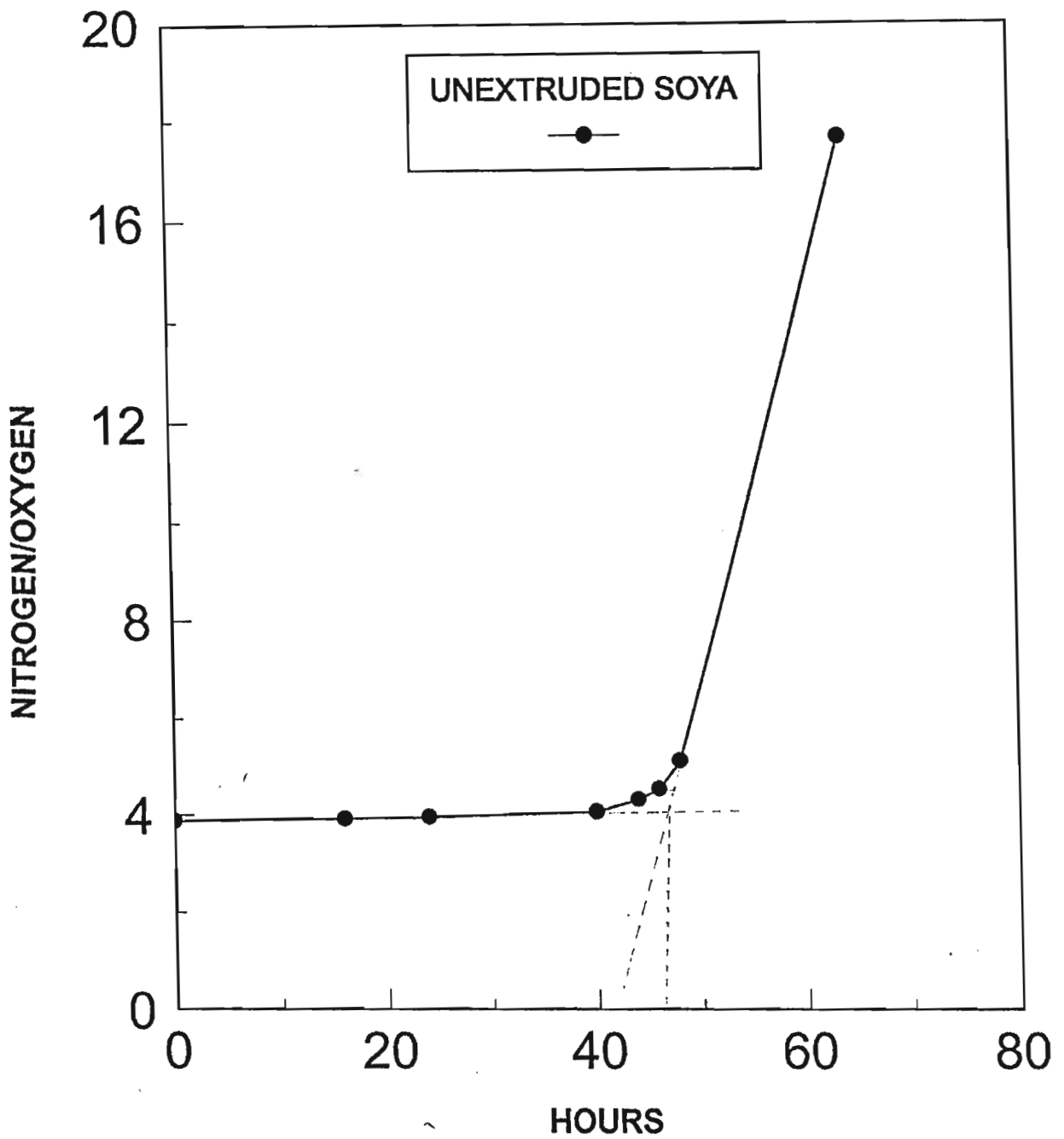


Figure 5.3a Oxidation of lipids of unextruded soya flour.

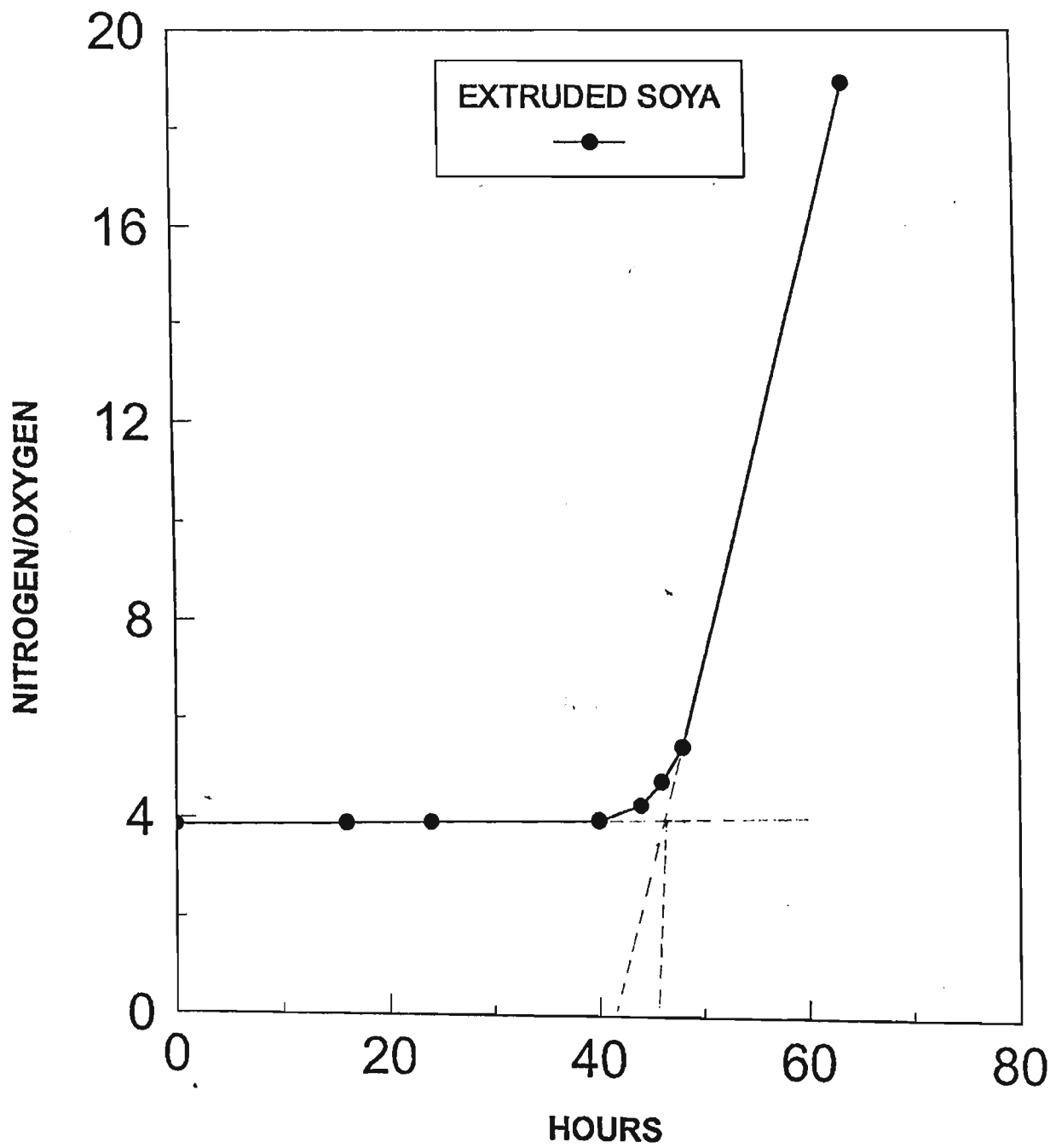


Figure 5.3b Oxidation of lipids of extruded soya flour.

Negligible differences were found between extruded and unextruded soya flours with respect to induction periods of the lipids, (Table 5.4), except that the extruded soya had a darker yellowish colour than unextruded soya.

Table 5.3 Extrusion Parameters of 5.3.2.1.(96)

Screw configuration

Pitch mm	50	50	50	33	25	25	35	35	25	-15
Length mm	200	100	100	100	100	50	100	100	100	50
type	DF	DF	DF	DF	SF	SF	SF	SF	SF	R
Gap mm										10

Aperture = 1 x 1.8mm I.D.

Thermocouples

temperatures

Heaters

Q1

Q2

Q3

Q4

Settings

62

90

200

160

Actual extrusion of std.soya flour

102

207

158

RPM of screws

80

Feed rate

37

Water addition

0

Table 5.4 Analysis of extruded and unextruded soya flours

Analysis	Unextruded soya	Extruded soya
Induction period hr	46.8	46.2
Available lysine g/100g protein	4.91	4.31
Reducing sugars %	0.31	0.80

The negligible difference between the induction periods of the extruded and unextruded soya indicates that no antioxidant compounds of significance were developed during extrusion. Notwithstanding this available lysine was reduced while reducing sugars increased, noticeably that free reducing sugars were being formed during extrusion. The loss in lysine probably results from the heat applied during extrusion. Also

the slight increase in reducing sugars could result from the shear effects of the extrusion process.

5.3.2.2 Extrusion of fullfat soya flour with reducing sugars.

The extrusions in this experiments varied with respect to the inclusion of simple sugars to standard soya flour prior to extrusion. Three soya flour mixes were extruded, one without any sugars, and the other two prepared with 9% glucose and 9% fructose respectively. The extrusion condition were kept constant but screw configuration changed to further increase shear and heat effects as listed in Table 5.5.

Visual examination of extrudates showed a variation in colour when compared with standard soya extrudate which was yellowish while glucose soya extrudate was brown in colour and fructose soya extrudate a very light brown in colour.

Samples of extrudate were cooled, sealed in aluminium foil pouches and stored at -16°C. Unextruded samples of the respective soya mixes were also stored under same condition.

Table 5.5 Extrusion parameters for 5.3.2.2. (96).

Screw configuration.

Pitch mm	50	50	50	33	35	25	25	15	15	15	-15
Length mm	200	100	100	100	100	100	50	50	100	50	50
Type	DF	DF	DF	DF	SF	SF	SF	SF	SF	SF	R
Gap mm											10

Aperture = 1 x 1.8mm I.D.

Thermocouples		temperatures		
Heaters		Q1	Q2	Q3 Q4
Settings		58	90	200 160
Actual	extrusion 1 std. soya flour		94	203 163
	extrusion 2 glucose soya flour		92	204 170
	extrusion 3 fructose soya flour		88	208 160
RPM of screws	80			
Feed rate	50 kg/hr			
Water addition	nil			

5.3.3 Oxidation studies by Ross and de Muelenaere method.

5.3.3.1 Oxidation of lipids from extruded soya samples.

The lipids were extracted from extruded soya samples of section 5.3.2.2 with n-hexane as per method described in 5.2.2.2. After solvent evaporation the lipids were stored in reaction vials under nitrogen at -16°C .

The induction period of the respective lipids from the extruded samples was determined by Ross and de Muelenaere method. Table 5.6 shows the N_2/O_2 ratios obtained from the oxidation of the lipids extracted from the different extrudates. The induction periods of the lipids were calculated from the oxidation curves in Figure 5.4, Figure 5.5 and Figure 5.6 and listed in Table 5.8.

Table 5.6 Nitrogen to Oxygen ratios calculated for lipids of extruded soya samples.

Hours	Extruded Soya as is	Extruded Glucose	Extruded Fructose
	N_2/O_2	N_2/O_2	N_2/O_2
0	3.88	3.86	3.89
24	3.92	3.90	3.95
48	4.19	4.04	4.18
52	5.35	4.10	4.33
53	7.05	4.14	4.66
54	8.80	4.17	5.13
55	11.94	4.21	5.89
56	-	4.25	7.15
72	-	4.96	-
74	-	5.25	-
76	-	5.99	-
78	-	6.75	-
80	-	7.88	-

The above N_2/O_2 ratios were plotted against hours and the following oxidation curves recorded.

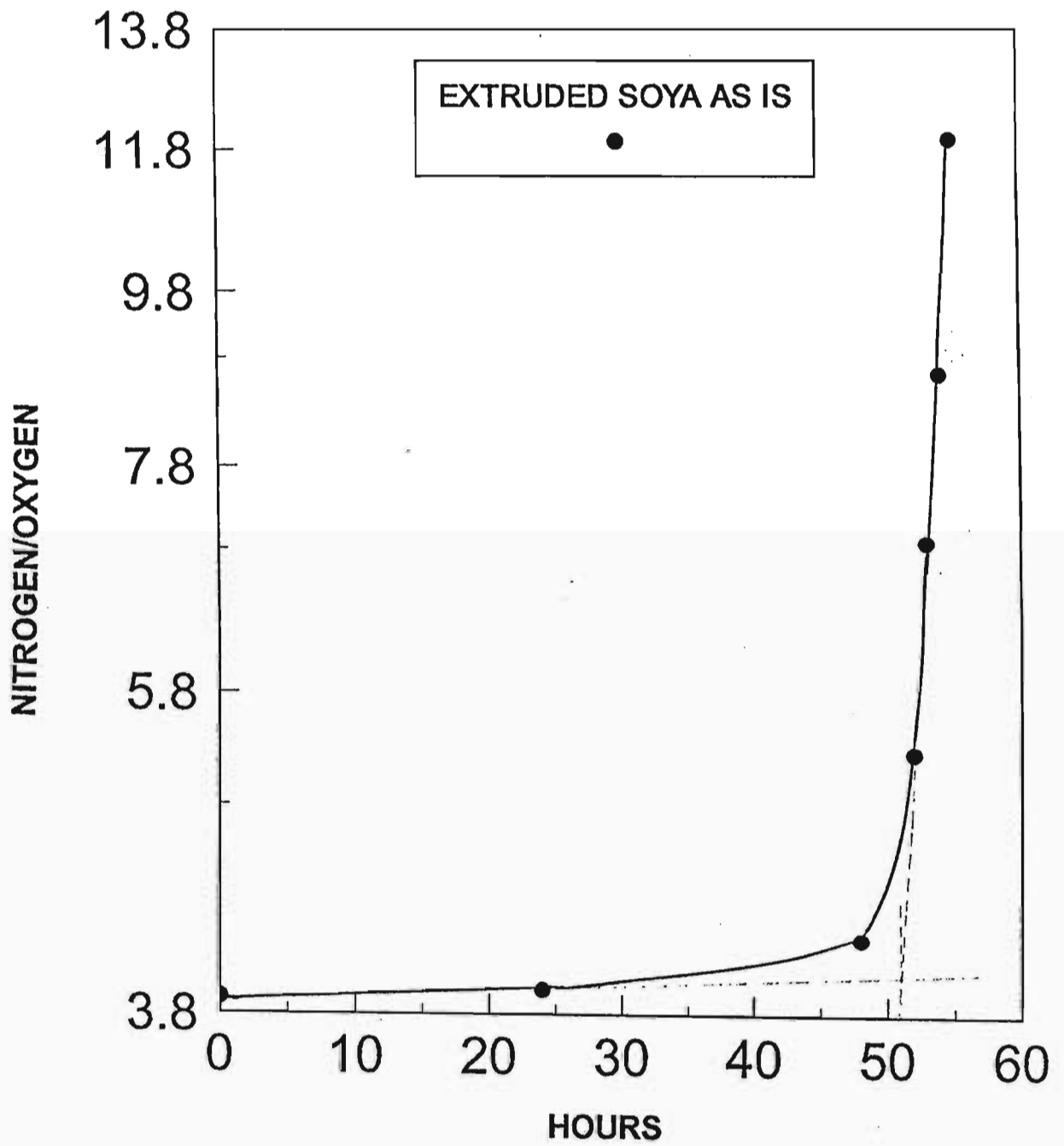


Figure 5.4 Oxidation curve for extruded soya lipids.

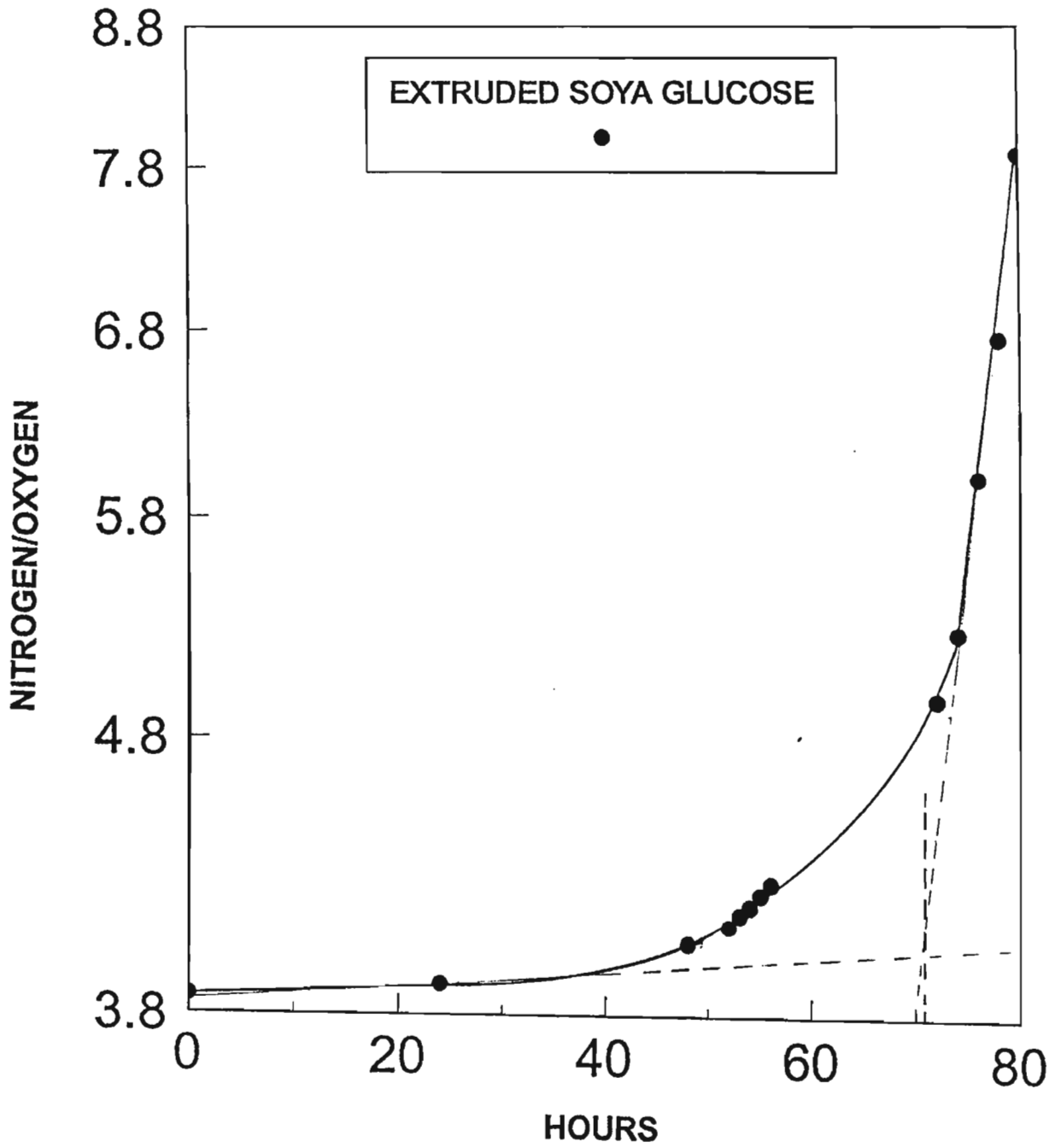


Figure 5.5 Oxidation curve for extruded glucose soya lipids.

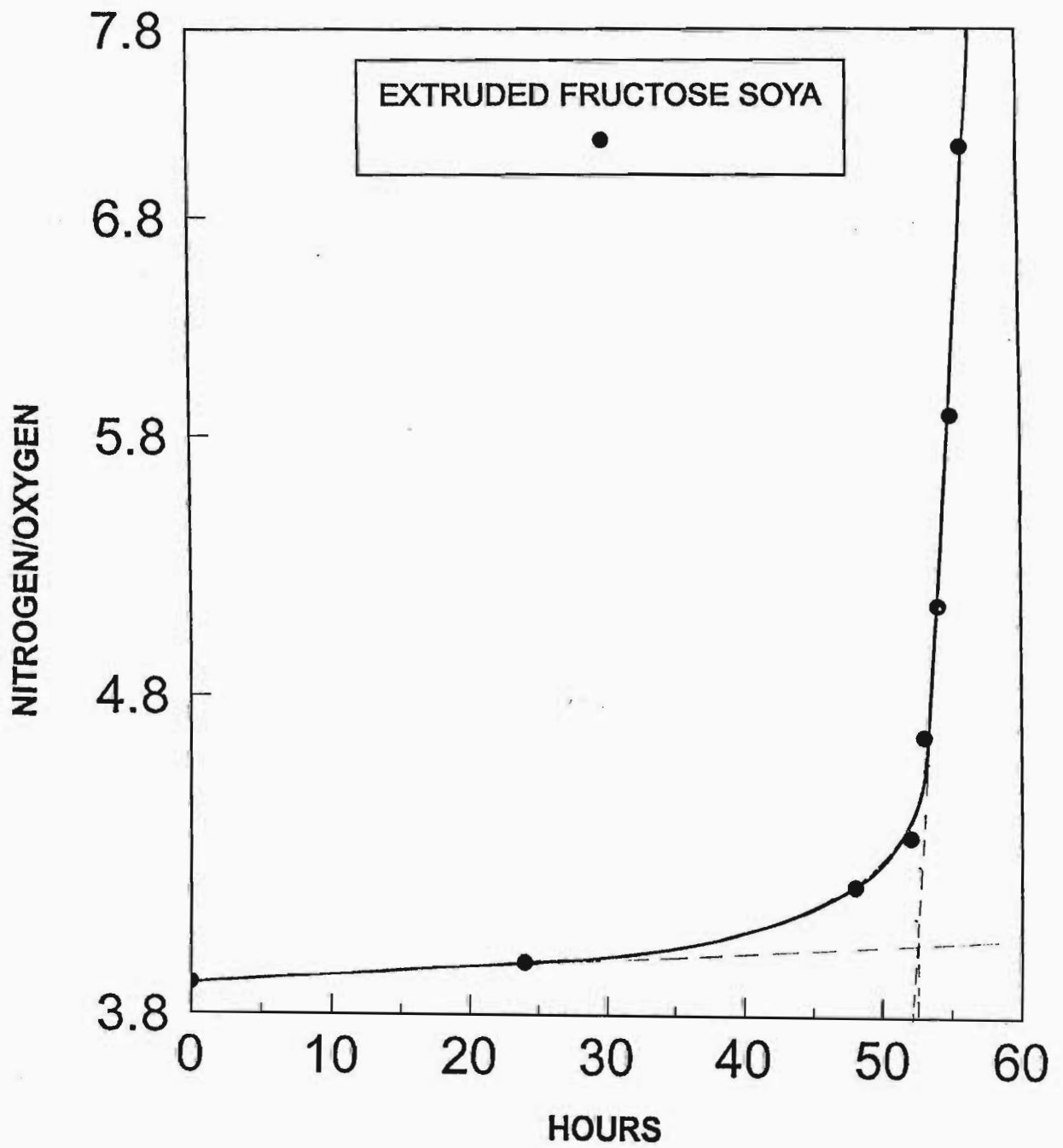


Figure 5.6 Oxidation curve for extruded fructose soya lipids.

5.3.3.2 Oxidation of lipids extracted from unextruded soya mixes.

The lipids were extracted from the unextruded soya mixes as prepared under section 5.3.2.2 with n-hexane as per method described in 5.2.2.2. After solvent evaporation the lipids were stored in reaction vials under nitrogen at 16°C.

The induction period of the lipids extracted from the unextruded soya mixes were determined by the Ross and de Muelenaere method. Table 5.7 shows the changes in N_2/O_2 ratios obtained from the oxidation of the lipids. The induction period of the lipids obtained from the oxidation curves in Figure 5.7 are listed in Table 5.8.

Table 5.7 Nitrogen to oxygen ratios calculated for lipids extracted from unextruded soya mixes

Hours	Extruded Soya as is	Extruded Glucose	Extruded Fructose
	N_2/O_2	N_2/O_2	N_2/O_2
0	3.86	3.85	3.88
24	3.91	3.93	3.90
48	4.03	4.10	4.06
51	4.80	4.40	4.50
52	6.22	6.40	6.30
53	8.06	7.88	8.18

The N_2/O_2 ratios were plotted against hours and the oxidation curves obtained as per Figures 5.7a, b, and c.

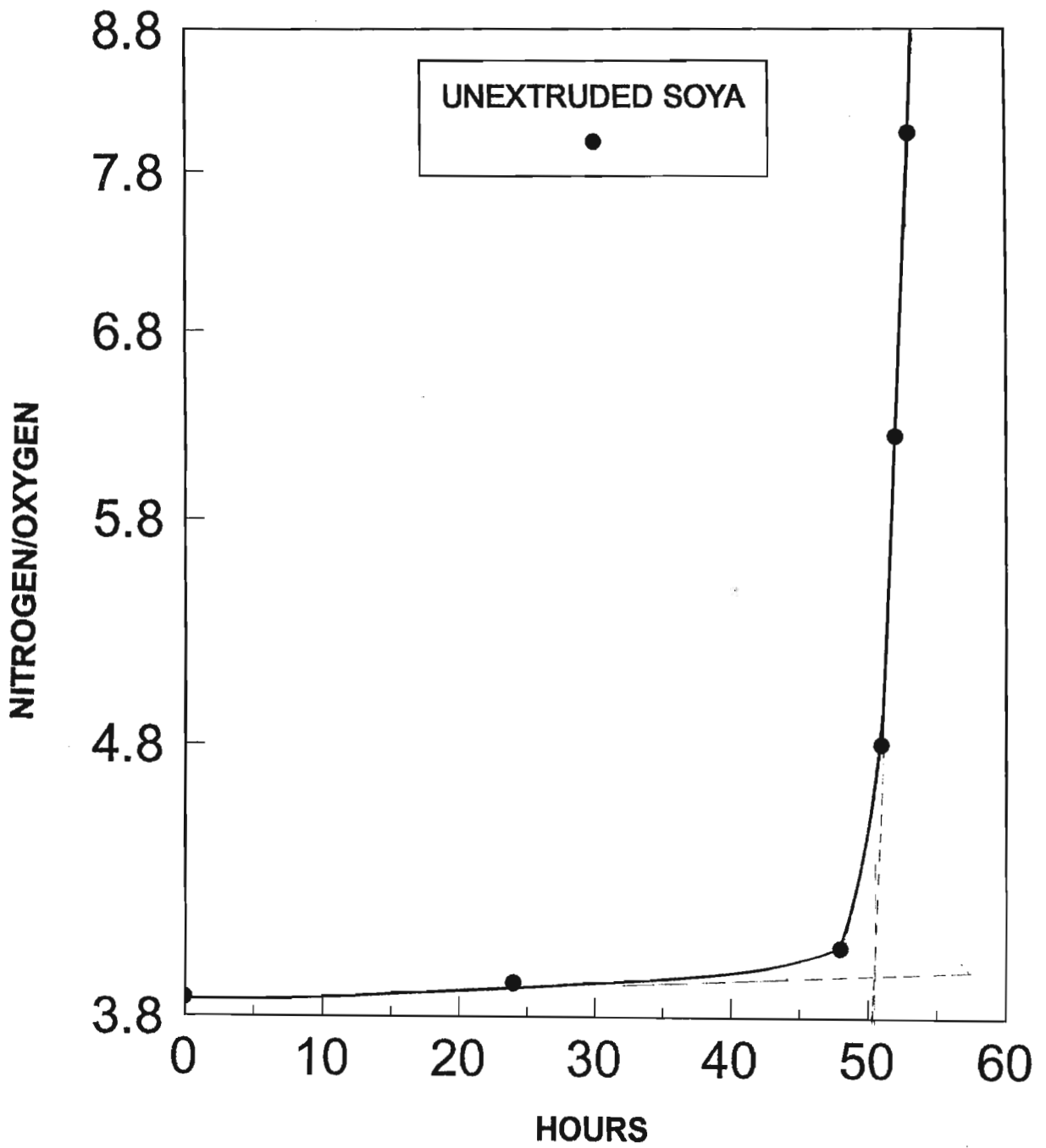


Figure 5.7a Oxidation curves for lipids of unextruded soya as is.

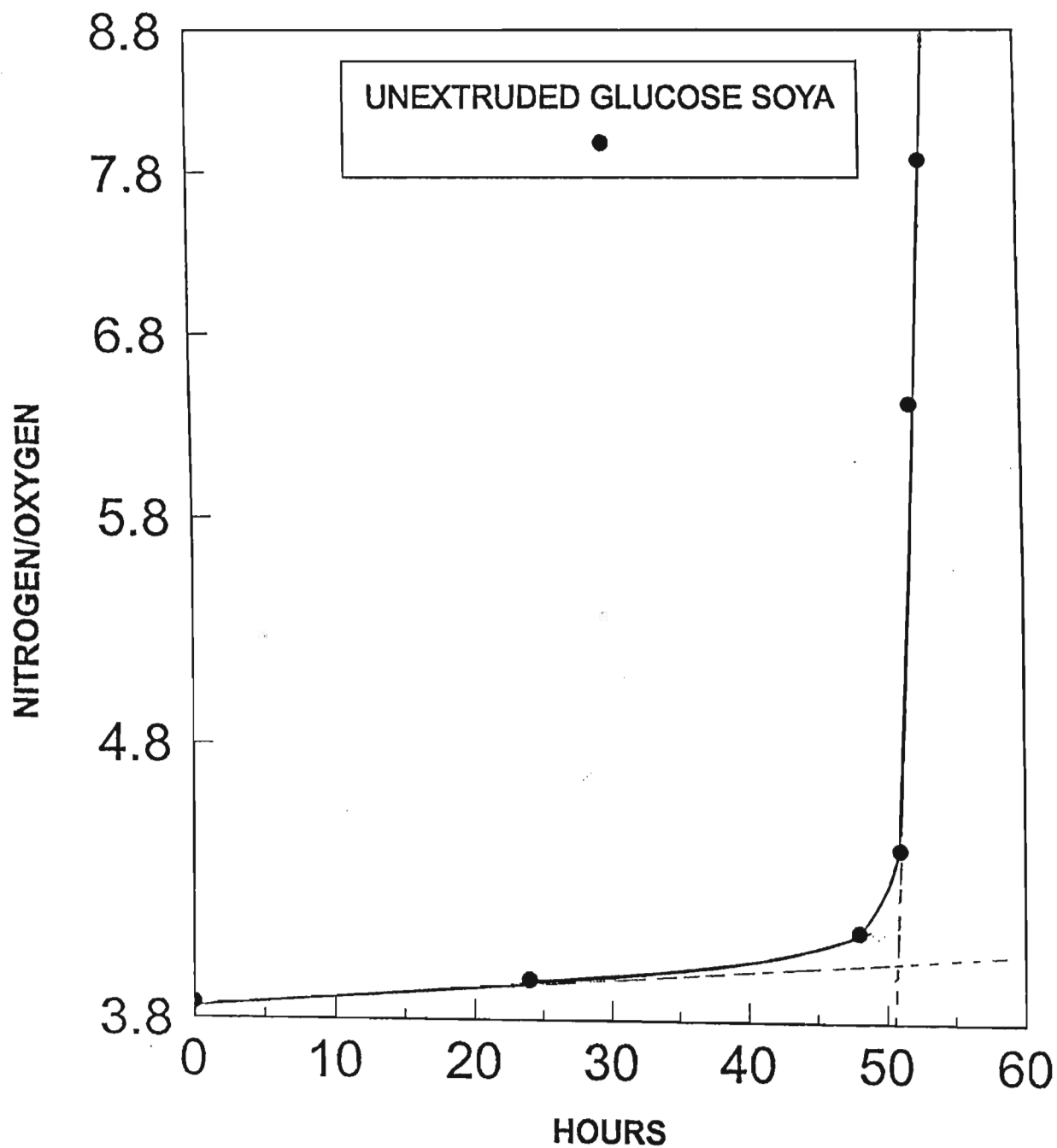


Figure 5.7b Oxidation curves for lipids of unextruded glucose soya.

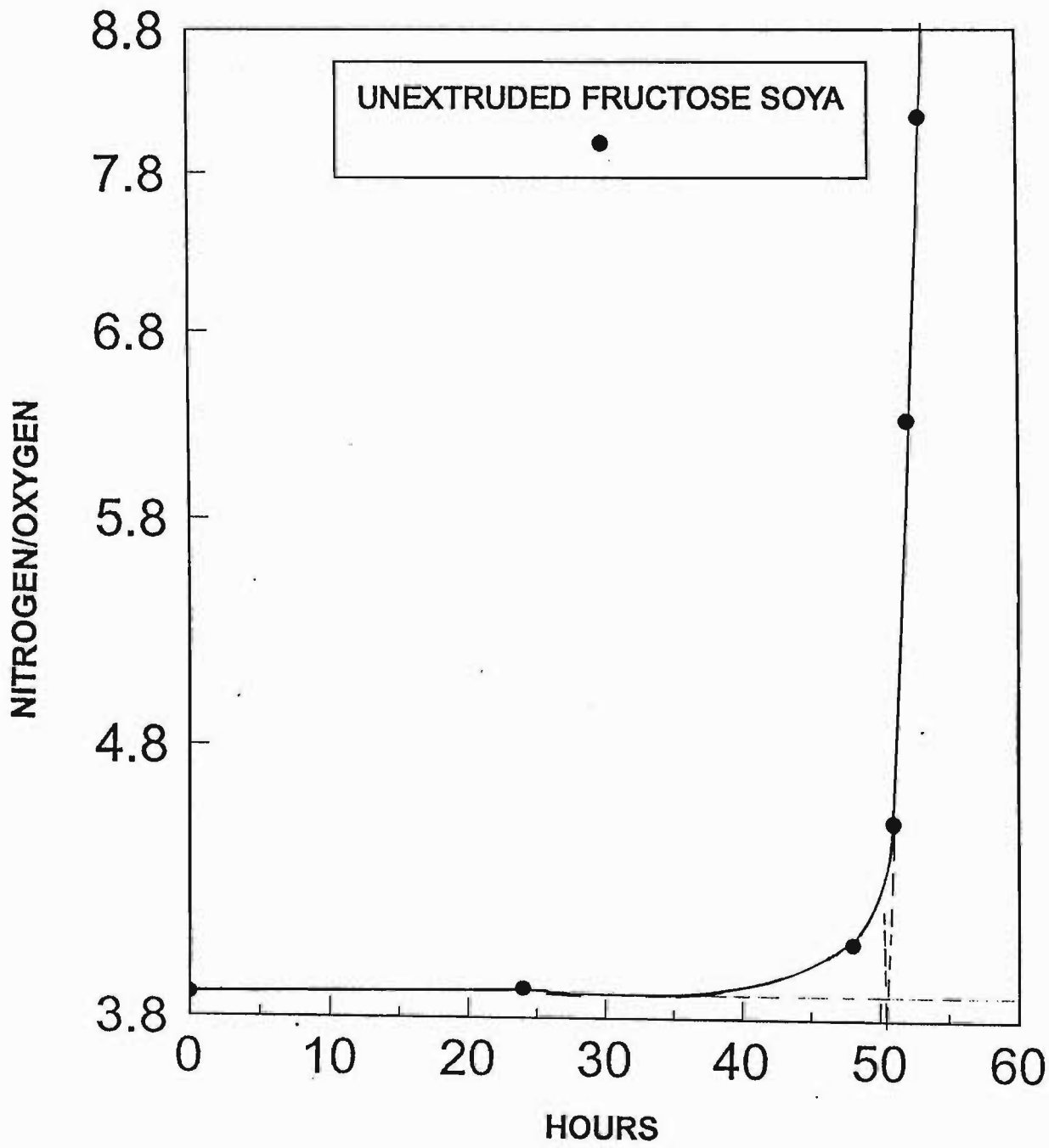


Figure 5.7c Oxidation curves for lipids of unextruded fructose soya.

Table 5.8 Induction periods of lipids extracted from extruded and unextruded soya mixes.

Samples	Induction periods (hr)	
	Unextruded	Extruded
Soya flour as is	50.8	51.7
Soya flour with glucose	50.8	71.1
Soya flour with fructose	50.8	52.5

The induction period of the lipids from unextruded soya samples are identical indicating that the presence of sugars in the mixes has no effect on the lipids. In the case of the extruded samples the presence of glucose in the extrusion mix resulted in an increased induction period of the lipids of 19.4 hours. The extruded sample with fructose in the extrusion mix increased the induction period by only 0.8 hours. The increased induction periods appears to be an indication that Maillard reaction products formed during extrusion due in presence of glucose and fructose are hexane extractable and hence contribute to antioxidant properties of the extracted oil.

5.3.3.3 Oxidation of unextruded and extruded soya mixes

The oxidation of unextruded flours and extruded full fat soya mixes with and without sugars was monitored by the Ross de Muelenaere method. The sample size used was 1.5g so as to maintain similar lipid content as used for the oxidation of lipids described in Chapter 3, method 3.2.2.1.

The unextruded soya mixes and extruded soya samples without any sugars showed a slight rise in nitrogen/oxygen ratio from 3.98 to 4.71 after 3 to 4 days while a gradual increase between 3.98 to 4.09 was noticed with unextruded and extruded samples containing sugars, and no distinct rise in nitrogen to oxygen ratio was observed after 7 days. A distinct increase in discolouration was observed in both the unextruded and

extruded samples containing sugars after 16 hours and continued to darken as storage period increased, indicating that Maillard reaction products were formed. This exercise was discontinued as the residual free sugars were reacting with the free amino groups and appeared to form brown Maillard reaction products under the experimental temperature (80°C) when the N₂/O₂ ratios was being measured. It has been cited in literature that such reaction between water soluble reducing sugars and amino acids occur in foods with water activity above the monolayer (0.3-0.7 range). The Maillard reaction is more heat sensitive than the lipid oxidation reaction. The activation energy for the Maillard reaction is in the 20 to 50 Kcal/per mole range compared to 15 to 25 Kcal/per mole for lipid oxidation (97). This difference in heat sensitivity presents a problem for accelerated testing of intermediate moisture foods. It has been reported that lipid oxidation in the case of dehydrated potatoes is the main deterioration reaction at 20°C. The Maillard reaction at this temperature does not present a problem. However, when the storage temperature increases, the rate of the Maillard reaction is accelerated two or three times compared to the lipid oxidation (97). In this case the antioxidant effect of the Maillard reaction produced during N₂/O₂ determinations will inhibit the lipid oxidation. This artifact phenomenon appears to have arisen under the present experiment with fullfat soyabeans.

5.3.4 **Oxidation Studies Rancimat Method.**

5.3.4.1 **Oxidation of lipids extracted from extruded and unextruded soya samples.**

Induction periods calculated from the oxidation of lipids from extruded and unextruded flours with and without sugars by the Rancimat Method is tabulated in Table 5.9.

Table 5.9 Oxidation of lipids extracted from extruded and unextruded soya samples.

Lipid samples	Induction periods (hr)	
	Unextruded	Extruded
Soya flour as is	276	264
Soya flour with glucose	414	258
Soya flour with fructose	294	246

The lipids from glucose and fructose soya extrudates showed an increase in induction period of 138 and 18 minutes respectively as compared to extruded soya flour as is, while no appreciable differences in induction period were noted for the lipids of unextruded soya mixes and extruded soya flour as is.

5.3.4.2 Influence of fullfat and defatted soya extrudates and TBHQ on oxidation of stripped sunflower oil.

The following experiment was conducted to establish whether defatted extrudates possessed any antioxidant activity. The extruded soya samples were defatted with n-hexane as per method 5.2.2.2 and the defatted residues were dried to remove residual solvent in a vacuum oven at 50°C for 16 hours. The brown pigments formed in the sugar extrudates were retained in the defatted extruded residue. The sample size for Rancimat test was increased to 2.5g for fullfat soya extrudates and 2.1g for defatted soya extrudates to account for the lipid content difference. The samples were dispersed into the stripped sunflower oil substrate used in Chapter 4 and oxidation of such was monitored on the Rancimat. Control sample of stripped oil substrate and a standard antioxidant i.e. TBHQ 200ppm in stripped oil substrate was also tested. The induction period of the respective samples are listed in Table 5.10

Table 5.10 Influence on induction periods of defatted and fullfat soya extrudates and TBHQ on stripped sunflower oil substrate.

Samples	Induction periods (minutes)	
	Fullfat	Defatted
Soya extrudate	30	21
Soya glucose extrudate	195	21
Soya fructose extrudate	36	21
Control oil substrate	18	-
200 ppm TBHQ in oil substrate	429	-

The low induction period recorded for the defatted soya extrudates, especially the soya glucose extrudate indicates that the components responsible for increased induction period as detected in fullfat soya glucose extrudate have been removed by hexane extraction, as illustrated in section 5.3.3.1 under oxidation of lipids from extruded soya samples. Fullfat Soya glucose extrudate shows increased induction period when compared to fullfat soya extrudate and equated to approximately 86 ppm TBHQ when compared to induction period of 200 ppm TBHQ (Table 5.11). A very small increase in induction period is noted for soya fructose extrudate equal to approximately 9 ppm TBHQ (Table 5.11).

Table 5.11 Calculation of Antioxidant activity of soya extrudates with respect to TBHQ.

Samples	Induction Period of Sample minutes	Induction period of stripped Oil minutes	Increased induction period of sample minutes	Increased induction period equated to TBHQ (ppm)
200 ppm TBHQ	429	18	411	200
Soya glucose extrudate	195	18	177	86
Soya fructose extrudate	36	18	18	9

5.3.4.3 Summary of Oxidation of lipids from soya extrudates.

Table 5.12 summarises the induction periods obtained by the Ross and de Muelenaere and Rancimat methods and the estimated percentage increase of induction period observed. The results also illustrate the effect of glucose and fructose during soyabean extrusion

Table 5.12 Summary of oxidation of lipids from soya extrudates.

Oxidation methods Samples	Ross & de Muelenaere I.P. (hrs)	Rancimat I.P. (min)
Soya glucose extrudate	71.1	414
Soya extrudate	51.7	276
Increased I.P.	19.4	138
% Increased I.P.	37.5	50
Soya fructose extrudate	52.5	294
Soya extrudate	51.7	276
Increased I.P.	0.8	18
% Increased I.P.	1.5	6.5

5.3.4.4 Determination of reducing sugars of extruded and unextruded soya.

The Fehlings Method for estimation of total reducing sugars was used to determine the reducing sugars in extruded and unextruded soya flours. The HPLC method was used to determine glucose and fructose content of the extruded and unextruded soya flours. The individual sugars were specifically analyzed to eliminate any reducing compounds arising from the formation of Maillard reaction products that would otherwise have been measured by the Fehlings Method. Table 5.13 compares the sugar results obtained by the two methods and expressed on dry basis.

Table 5.13 Analysis of sugars by Fehlings and HPLC method.

Samples	% Total reducing sugars by Fehlings method	% Glucose by HPLC	%Fructose by HPLC
Extruded soya flour	0.32	Trace*	nil
Extruded glucose soya flour	3.99	3.22	nil
Extruded fructose soya flour	7.30	Trace*	7.13
Unextruded soya flour	0.86	Trace*	nil
Unextruded glucose soya flour	9.16	9.44	nil
Unextruded fructose soya flour	8.86	Trace*	8.99

*Small glucose peak present in chromatogram but not integrated.

Both the Fehlings method for reducing sugars and HPLC method for glucose and fructose analysis gave a good estimation of the sugars present in the soya samples which theoretically contained about 9.1%. The HPLC analysis eliminates any over estimation of reducing sugars that may be measured by the Fehlings method as a consequence of reducing compounds formed by the Maillard reaction. Comparison of glucose in the extruded and unextruded soya glucose samples shows that 66% of glucose is utilised in the formation of Maillard reaction products. As for fructose content in the extruded and unextruded soya samples approximately 21% fructose is utilised in Maillard reaction formation during extrusion processing.

5.3.4.5 Determination of available lysine in extruded and unextruded soya samples.

Available lysine was determined on the extruded and extruded soya samples to establish the loss of available lysine by the formation of Maillard reaction products during extrusion. Table 5.14 lists the available lysine, moisture and protein content of the respective soya samples.

Table 5.14 Moisture, protein and available lysine content of soya samples.

Samples	% Moisture	% Protein	Available lysine g/100g protein
Extruded soya	2.87	34.42	4.28
Extruded glucose soya	2.66	31.54	1.51
Extruded fructose soya	2.71	31.18	3.78
Unextruded soya	7.93	33.03	4.91

Comparison of unextruded and extruded soya shows that only 13% available lysine is lost during the mild extrusion process. The addition of glucose or fructose to the extrusion mix results in the loss of 69% for soya glucose extrudates and 23% for soya fructose extrudate. These losses of available lysine result from the formation of Maillard reaction products.

5.4 DISCUSSION

From the evaluation of several solvents suitable for lipid extractions, n-hexane was found to extract the maximum lipids from soyabean flour. In general, non-polar solvents such as hexane require cold crystallization and filtration temperatures than do semipolar solvents and is commonly used for commercial

fractionation of triglycerides (98). Hence, hexane was chosen for all subsequent lipid extractions carried out in this investigation.

It was established in the initial extrusion experiments that mild extrusion conditions had little effect on the induction periods in the extruded and unextruded soyabean samples. Although a slight loss of reducing sugars and available lysine was noticed in the extruded, there was no appreciable increase in the induction period of lipids extracted from extruded and unextruded soya samples. Most studies on the changes that proteins undergo during extrusion processing have been concentrated on the free amino group of lysine which decreases following extrusion (87). But no information is available on the effect of extrusion on prevention of lipid oxidation.

Extrusion of soyabean flour with incorporation of reducing sugars produced a brown extrudate with glucose and a light brown extrudate with fructose while plain soyabean extrudate was a deep yellowish extrudate. Oxidation studies of the lipids from extruded and unextruded soya mixes showed an appreciable increase in induction period of lipids extracted from the extruded glucose soya sample. Unextruded soya samples containing identical levels of reducing sugars showed no difference in induction periods. This indicates that the increased induction period results from the extrusion process and the addition of glucose to the extrusion mix. Soya extrudate without glucose had negligible effect on the induction period. Recent studies on heating model systems to temperatures equivalent to that in the extruder resulted in a marked decrease in glutamic acid residue. In the presence of small amounts of reducing sugars the decrease in glutamic acid levels is even more marked (45). In soya systems similar decreases are observed and the decrease is more marked in native flour than in dialysed flour containing little soluble low molecular weight carbohydrate material (99).

From oxidation studies on the unextruded soyabean mixes and extruded soya samples, a low induction period was noticed for both unextruded and extruded soya samples without sugars. For those samples which contained the reducing sugars, no oxidation was evident over a 7 day period, but a distinct

brown darkening of both the extruded and unextruded samples was observed from first day of storage. The darkening effect was very slow in the unextruded samples and on the extruded fructose soya. The gradual increase in brown pigment formation, indicates development of Maillard reaction products from unreacted residual sugars and amino groups present, resulting in longer induction periods. At this elevated temperature the rate of the Maillard reaction increases two or three times the lipid oxidation rate, hence the long induction period (97).

Oxidation studies conducted by the Rancimat method reconfirmed that lipids from the soya glucose extrudate had a longer induction period (that is, 138 minutes) than soya fructose extrudate (18 minutes). Oxidation of fullfat and defatted soya extrudates revealed that all the defatted soya extrudates had identical low induction periods while the fullfat soya glucose extrudate had a high induction period. These results indicate that the components responsible for increasing the induction period are removed by hexane extraction and retained in the lipids after evaporation of hexane, hence the higher induction period noted in the lipids of soya glucose extrudate.

Comparing the increased induction period of soya extrudates to induction period of TBHQ antioxidant, it was estimated that the antioxidant activity of soya glucose and soya fructose extrudates could be equated to 86 ppm and 9 ppm TBHQ respectively. The retention of brown pigments in the defatted residues with low induction period indicate that the brown pigments, developed by the Maillard reaction, possess no antioxidant activity. During the "advanced" stage of the Maillard reaction, polymerization of premelanoidins have been shown to form high molecular weight melanoidins, which were responsible for the brown pigments (100).

From the induction periods measured by the two methods used for oxidation studies, the lipids of soya glucose extrudates showed an increase in induction period of 37.5 to 50% respectively. Lipids of soya fructose extrudates showed an increase in induction period of 1.5 to 6.5% for the respective methods.

Analysis of glucose and fructose in the extruded and unextruded soya samples did show that 66% of glucose was utilised in the formation of the Maillard reaction products while for the fructose extrudates only 21% was utilised. Determination of available lysine revealed that 69% of available lysine from the soya glucose extrudate was lost to Maillard reaction and 23% was lost in the soya fructose extrudate.

The high losses of glucose and available lysine together with the development of dark brown colour in the soya glucose extrudate indicate the formation of Maillard reaction products. The increased induction period or antioxidant activity observed for the soya glucose extrudate are due to the formation of Maillard reaction products.

CHAPTER 6

DISCUSSION AND CONCLUSION

The Maillard reaction is primary of importance to the food manufacturer, since it is mainly responsible for the aromas and colours that are formed during heating or storage of food products. The ability to control this reaction is still very limited, although recent studies have indicated how it may be manipulated (39), particularly with regard to obtain desirable flavour, colour and aroma. On the other hand the Maillard reaction is sometimes undesirable, when dehydrated foods darken and develop off-flavours on storage.

Heating at intermediate temperatures (eg. 100°C), cooked or caramel flavours are produced, while at higher temperatures (150°C), 'toasted' or 'roasted' aromas are formed. It is clear that aroma profile varies with temperature and time of heating.

During preliminary studies leading up to MRP influence on oxidation and rancidity development, potentially similar oils were evaluated. Sunflower oil was identified as a suitable substrate for monitoring antioxidant activity studies as it possessed a short induction period, a high iodine value, hence a high unsaturated fatty acids content from which a high Oxidative Susceptibility value could be calculated. Another potential that is, methyl linoleate was also studied but when compared to sunflower oil it was costly and not easily available at short notice. Oxidation studies with methyl linoleate revealed that as a substrate it was very sensitive to oxidation and recorded varying induction periods over short storage spells. This indicated that it would not be suitable for prolonged storage exercises were minute traces of oxygen would affect its stability. Frankel (63) and Meyer (75) in their evaluation of antioxidants also reported similar problems when using emulsions of linoleic acid and methyl linoleate as substrates. They found significant differences in the performance of antioxidants tested between linoleic acid emulsions than with triacylglycerol emulsions.

In view of this a storage test exercise was conducted to establish suitable conditions for prolonged storage of sunflower oil to ensure that the performance of the substrate for monitoring antioxidant was not affected by storage. The exercise revealed that both vacuum and nitrogen headspace at 4°C are suitable. Nitrogen treatment was selected since it was found to be difficult to remove all traces of residual air diffused in the oil by vacuuming.

The two methods used to measure the induction period of extracted lipids indicate very similar results. The Ross and de Muelenaere method is a static system, in that it follows the change in nitrogen to oxygen ratio over a period of time. The Rancimat method is a dynamic method which accelerates oxidative deterioration by a continuous flow of air at elevated temperature. The products formed during oxidation process include volatile dicarboxylic acids leading to the change in electrical conductivity which is monitored (101). The Ross and de Muelenaere takes 1-7 days depending on oil type to obtain results and uses a small sample size (0.25g). The Rancimat method requires a larger sample size (2-4g) and results are obtained within 1-24hrs. This difference between the respective methods results from a different rate of deterioration which is brought about by the oil surface area exposed to atmosphere, and the depth of oil used and the temperature (101). Both extracted oil and soya flour substrates could be tested by the Rancimat method. In the case of the Ross and de Muelenaere method only oil samples can be tested successfully.

One of the drawbacks of the Rancimat method reported by Gordon and Mursi (101) which agrees with findings of Kochhar and Rossel (111) is that the Rancimat method under-estimates the stability of oil samples containing BHT and similar components, due to the volatility of the antioxidant at high temperature at which the Rancimat is operated. This effect is likely to be less in the case of the Ross and de Muelenaere method since measurement is carried out at lower temperature. Unlike the Rancimat method, the Ross and de Muelenaere is an enclosed system therefore eliminates any loss of BHT. However further investigation needed to establish such benefits. Frankel (53) has highlighted a recommended testing protocol for evaluating natural antioxidants and oxidative stability of foods such that conclusions reached in many oxidation studies could be validated.

Extrusion conditions were established whereby increased shear effects had very little effect on induction period of soya flour lipids as compared to unextruded soya flour lipids, while a slight loss of lysine and reducing sugars was evident. Addition of glucose or fructose to soya flour resulted in development of brown coloured extrudates which were not observed in the soya extrudates without added reducing sugars. It is evident from literature that nonenzymatic browning arises from Maillard reactions between ϵ -amino groups of protein bound lysine and aldose or ketose reducing sugars (102). Extrusion of soyameal report indicate losses in available lysine of up to 40% depending on extrusion conditions (103). In the present study 69% of lysine loss was established to occur.

No research has been published on the relationship between the antioxidant effect of MRP to the level of the Maillard reaction. From literature it would appear that discrepancies exist on the interrelationship between the extent of browning of Maillard reaction and antioxidant activity (104). Beckel and Waller (105) reported that the maximum antioxidant activity of MRP was achieved after 20hrs of sugar-amino acid heating while other studies showed that MRP produced in the early stages of the Maillard reaction had strong antioxidant activity (106). These conflicting reports regarding the stages of browning and their antioxidant activity may be due to varying experimental conditions (107), and that compounds formed by Maillard reaction can exhibit antioxidant properties with different modes of action (105).

In the present study oxidation of fullfat extruded and unextruded soya mixes with and without added sugars showed that the highest development of brown Maillard reaction products occurred in those mixes that contained sugars and had undergone extrusion. Similar extruded and unextruded soya mixes without sugars showed very slight Maillard browning and low induction periods. Matsuda *et al.* (112) reported development of brown Maillard reaction products when heating ovalbumin with glucose, mannose and galactose at 50°C over 0-10 days. The protein-galactose mixture was 2-3 times stronger in brown colour than any of the other sugar mixes. It was suggested that such a remarkable difference in the reaction rate was probably due to the stereochemistry of the sugars (112). Similar structure differences between glucose and fructose probably explains the higher browning maillard reaction in the soya glucose extrudate to the soya fructose extrudate.

Oxidation studies by the Rancimat method identified lipids from soya glucose extrudates to have longer induction periods than lipids from soya fructose extrudate. Oxidation of fullfat and defatted soya extrudates showed that all defatted soya extrudates had similar low induction periods while fullfat soya glucose extrudate had a high induction.

Products responsible for the increased induction period in the soya glucose extrudate were shown to be removed by hexane extraction. This was confirmed by low induction period observed for defatted soya glucose extrudate. The brown pigments developed during extrusion were found to be retained in the defatted soya glucose extrudate, which from oxidation studies possessed very low induction period. This suggests that antioxidant activity is independent of pigment formation. Furthermore experimental evidence leads to the fact that brown pigments are probably formed by ionic condensation of primary amino groups of protein with conjugated unsaturated aldehydes (8). Such complex compounds are not as easily extractable as smaller molecules (52). It is of significance to note that the brown pigments retained in the defatted product with low induction period indicate that such pigments possessed no antioxidant activity.

Recent studies indicated that Maillard reaction volatiles (MRV) products of low molecular weight possess antioxidant activity. The maximum antioxidant effect was obtained with volatiles from 12-18hrs of heating a glucose-glycine solution (104). While melanoidins were the ultimate products of the Maillard reaction, many compounds of low molecular weight, which play an important role in flavour and off-flavour production, are also formed (52). Shibamoto and Eiserich also evaluated several volatile heterocyclic compounds formed by Maillard reactions in tocopherol-stripped corn oil that possessed antioxidant activity (108). Many of these compounds are lipolytic and possess reducing and chelating properties as well as act as electron traps or hydrogen donors (51). A fractionation study of brown Maillard reaction products on Sephadex columns indicated that low molecular products significantly contributed to overall antioxidant activity. The opinion of Eichner (52) and others seems to be that colourless intermediates of the Maillard reaction are essentially contributing to the antioxidative effect of Maillard reaction products (52). The fact that antioxidant activity has been found to be extracted by hexane indicates that the

antioxidant compounds are low molecular weight products formed by the Maillard reaction, and such is consistent to some earlier published work.

Comparison of induction periods of soya glucose and soya fructose extrudates to induction period of TBHQ antioxidant (200 ppm) gave an antioxidant activity of 86 ppm and 9 ppm for soya glucose extrudate and soya fructose extrudate respectively. Overall increased induction period measured by the two methods employed for soya glucose extrudate ranged between 37.5% to 50%. In a study of antioxidant activities of Maillard reaction products formed during blanching, dehydration, and roasting of peanuts at 160°C for 90 minutes, antioxidant activity was found to be close to that of 200 ppm TBHQ (109).

Analysis of residual sugars and available lysine in extruded and unextruded soya glucose samples indicated that 66% of reducing sugar (glucose) and 69% available lysine were utilised in the formation of Maillard reaction products. The ratio of reactants between glucose and lysine in the Maillard reaction being 1:1 and the excess lost lysine is probably utilised in covalent bonding with other protein groups. Similarly, 21% reducing sugars and 23% available lysine were utilised in the Maillard reaction of soya fructose extrudate.

Analysis of reducing sugars, glucose and fructose clearly indicate that glucose is more reactive than fructose in the formation of Maillard reaction products in conjunction with available lysine present in the extrudate. Although cited in literature that fructose is about one-tenth as reactive as glucose (110), it was found in this study to be one-third as reactive as glucose. The probable reason for this difference stems from varied experimental conditions.

Maillard reaction products (MRP) are widespread in processed foods and have received much attention as antioxidants, but no work has been published yet on the antioxidant effects of Maillard reaction products produced during extrusion. Ledward and Tester (45) have indicated that most studies of the changes that protein undergoes during extrusion processing have been concentrated on the free amino group of lysine which decreases following extrusion.

Furthermore it has been suggested that the loss of lysine is also important in developing the structure of the extruded product, since covalent bonds so formed would be very strong (45).

The results presented in this study highlights the Maillard reaction products as sources of natural antioxidants. It is in line with other publications that antioxidants result from lysine and reducing sugars and that glucose MRP possess strong antioxidant activity. From a detailed literature survey, it is evident that the data presented here are the first to establish that such antioxidant properties can be obtained during mild extrusion of soyabeans in the presence of glucose.

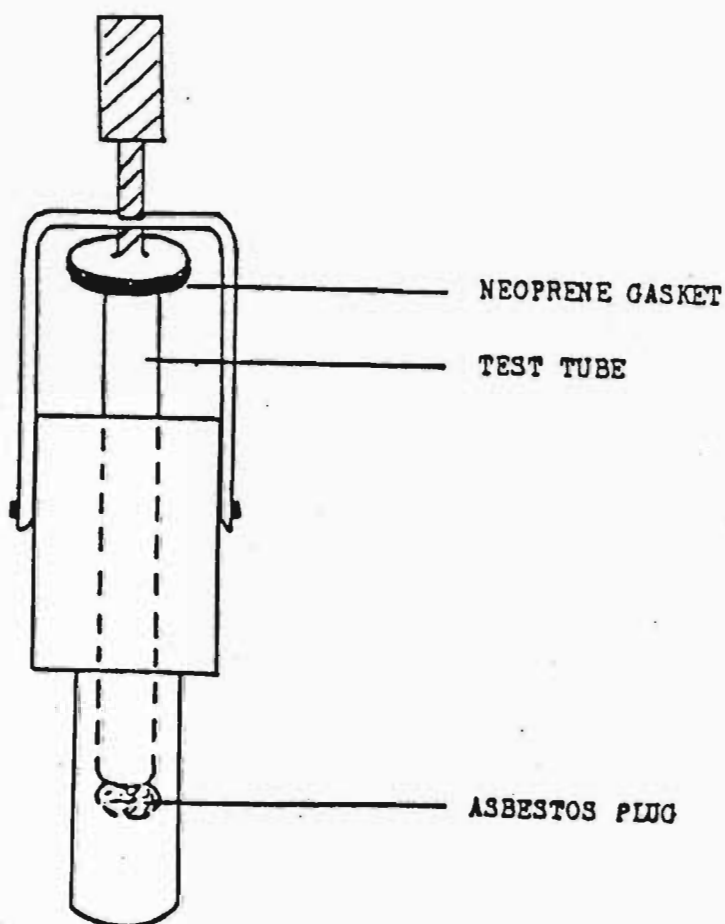
Based on this study, further investigations would be of considerable interest: such as the effect of varying levels of glucose, addition of foods containing reducing sugars such as milk, and eventual identification of hexane extractable compounds responsible for antioxidant properties.

Finally the numerous ways in which natural preservatives interact with food constituents and with each other to inhibit oxidation suggests that antioxidant systems may be customized to specific food compositions by designing suitable processing based on their pro-oxidant constituents and their natural antioxidant activities.

APPENDIX 1

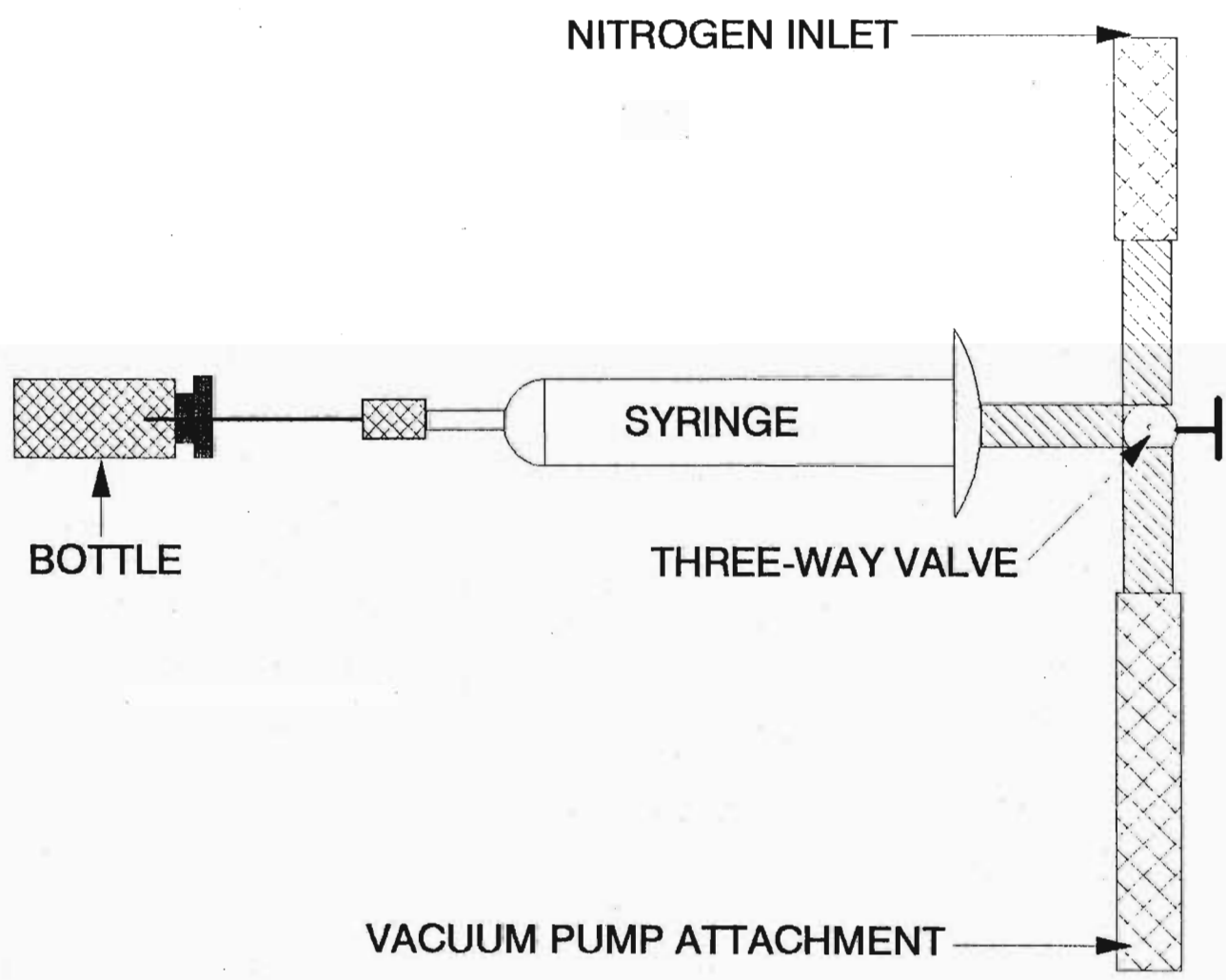
Apparatus

- (i) Heating block which will maintain a temperature of $170 - 180^{\circ}\text{C}$.
- (ii) Bomb apparatus, as shown below, with small test tubes and protection against explosion.



Schematic diagram of the bomb apparatus.

APPENDIX 2



Schematic diagram of a three way valve system with attached syringe.

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